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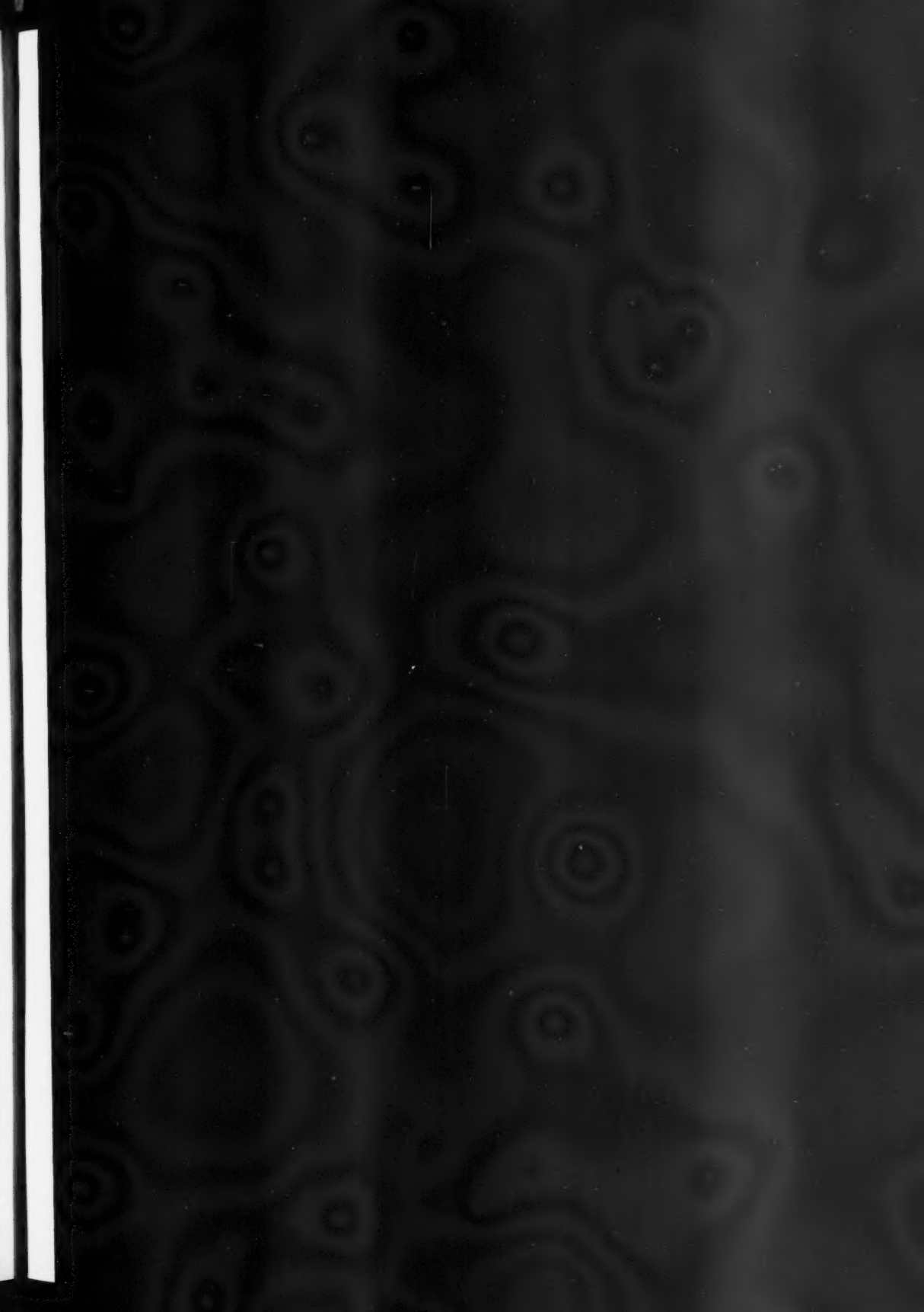

ENDOCRINOLOGY

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THE DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF THE RAT AFTER THE ADMINISTRATION OF RADIOACTIVE GROWTH HORMONE PREPARATIONS[1,2]

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The Sloan-Kettering Institute of the Memorial Center for Cancer and Allied Diseases, New York, New York

IT IS not known with certainty whether growth hormone acts via a specific target gland, or directly on peripheral tissues, or both. The response of rats to injections of growth hormone preparations were manifest despite either gonadectomy (van Wagenen, 1928), adrenalectomy (Simpson, Marx, Becks, and Evans, 1944), or thyroidectomy (Scow and Marx, 1945). Moreover, pancreatectomized cats (Milman, DeMoor, and Lukens, 1951), or pancreatectomized, adrenalectomized, thyroidectomized dogs (Gaebler and Robinson, 1942) maintained on insulin were able to store additional nitrogen after the administration of growth hormone.

The recent success in labeling anterior pituitary preparations with radioactive isotopes (Sonenberg, Keston, and Money, 1951a and 1951b; Sonenberg, Money, Keston, Fitzgerald, and Godwin, 1951; Ferrabee, Johnson, Mithoefer, and Gardella, 1951; Cox, 1951; Sonenberg, 1952; Sonenberg,

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[1] This project was supported in part by grants-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by a grant from the Atomic Energy Commission AT (30-1) 910.

[2] Presented in part at the 1952 meeting of the Endocrine Society in Chicago.

[3] Scholar of the American Cancer Society.

[4] This work was partly carried out during a tenure of a fellowship in Cancer Research of the American Cancer Society and as a Damon Runyon Senior Clinical Research Fellow.

Keston, Money, and Rawson, 1952), has offered an opportunity to define with greater precision the possible sites of localization of labeled hormone preparations after administration to experimental animals. This report presents the results of studies with radioiodine labeled growth hormone preparations under various experimental conditions.

MATERIAL AND METHODS

A. *Chemical*

The same growth hormone preparation[5] (hereafter referred to as growth hormone) was used throughout this study. This preparation was labeled in different lots under the same stoichiometric conditions. (There was good agreement between the various lots of yields and resulting molecular ratios.) The techniques employed in labeling growth hormone as well as other hormone and non-hormone preparations were similar to those reported earlier (Sonenberg, Keston and Money, 1951a) except that after iodination of the growth hormone, it was necessary to dialyse against isotonic saline which had been made minimally alkaline with a bicarbonate-carbonate buffer. (The growth hormone preparation was insoluble in isotonic saline alone.)

B. *Biological*

For assay of growth hormone activity, the technique of body weight increase of hypophysectomized rats (Marx, Simpson, and Evans, 1942) was used. Ten female rats that had been hypophysectomized two weeks prior to the assay were used in each group for the determination of biological activity. Each animal was injected subcutaneously daily for 14 days with 50 μg. of labeled or unlabeled growth hormone in 1 cc. of isotonic saline. A control group of animals received 1 cc. of isotonic saline daily by subcutaneous injection.

In the localization studies, both male and female Sprague-Dawley and Wistar rats were employed. (In the data reported only Sprague-Dawley animals were used. No differences were noted between these two strains.) The animals ranged in weight from 50 to 350 gm., but for each individual experiment the body weights were quite uniform. Unless otherwise indicated, comparisons have been made only between groups of animals of comparable weight. Approximately 160 animals received the radioactive growth hormone while I^{131}-labeled control substances were administered to approximately 300 animals. (For the sake of brevity, all the experiments are not tabulated in this report. There were fluctuations between experiments, as the reported data indicate. However, there was sufficient reproducibility to consider the reported data as representative.)

Although the majority of the animals employed in the distribution studies were intact, several experiments were conducted on rats that had been hypophysectomized at varying periods of time prior to the administration of the radioactive materials.

Other animals were pretreated with one injection of alloxan (25 mg./kg. body weight intravenously) 2 to 3 weeks prior to the administration of the radioactive preparations. Another group of animals was pretreated with dl-ethionine (420 mg./kg. intraperitoneal-

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[5] We should like to thank the Armour Laboratories for making this preparation available to us.

In this preparation (Armour 22 KR2) thyrotrophic activity was present to the extent of 0.03 to 0.04 unit per milligram (5–10% of Armour 2R3). No gonadotrophic, adrenocorticotrophic, prolactin, or posterior pituitary activity was found on assays performed by the Armour Laboratories.

ly per day for 14 days). At the time of sacrifice the pancreases from the normal, alloxanized and ethionine-treated animals were placed in Bouin's solution and fixed for histological examination.[6] (All animals that received alloxan were profoundly glycosuric although no attempt was made to quantitate the extent of glycosuria or hyperglycemia.)

Radioactively labeled preparations (growth hormone or control) were injected intracardially unless otherwise noted, into the rats while they were under ether anesthesia. Each animal received approximately 100 μg. of growth hormone containing 25 to 100 microcuries of radioactive iodine. The animals were sacrificed by exsanguination from the dorsal aorta at varying times after injection (3, 10, 30, 100 minutes, 5 and 24 hours). Representative tissues were removed, weighed and placed in 1 cc. of a 0.1 N NaOH solution. The tissues were digested for 24 hours at 80° C. The radioactivity was determined by gamma ray measurement in a multiple center wire counter (Marinelli and Hill, 1948), or in a well-type scintillation counter. Some tissue homogenates were transferred to steel cup planchettes, dried under an infra-red lamp and the radioactivity assayed with an end window Geiger-Mueller counter.

EXPERIMENTAL RESULTS

The biological activity of radioiodine labeled growth hormone, as measured by an increase in the weight of hypophysectomized rats, has been largely retained as shown in Figure 1. This preparation was labeled with an iodine to protein molecular ratio of 3.5 to 1. To determine the iodine to protein molecular ratio, the assumption was made that the growth hormone preparation represented a homogenous protein of molecular weight of 44,000 (Li and Moskowitz, 1949). Other growth hormone preparations that were similarly treated including dialysis but with omission of the iodination step, showed no loss in ability to promote weight gain in hypophysectomized animals.

After an intracardiac injection of radioactive growth hormone to the intact rat, radioactivity could be detected in most of the tissues examined (Table 1). Tissues with concentrations of radioactivity greater than blood were the pancreas, adrenal, liver, spleen, kidney, thyroid and thymus. The concentration of radioactivity in the pancreas was greatest 1 hour after an intracardiac injection (Fig. 2), following which the radioactivity fell, but significant concentrations still remained at 24 hours. During the first hour, the concentration of I^{131} in blood fell at a more rapid rate than the concentration in the pancreas. After one hour there was a slower loss of radioactivity from blood than from pancreas.

When I^{131}-labeled inorganic iodide was administered along with unlabeled growth hormone, the concentration of radioactivity in the pancreas and the organ/blood ratio were significantly lower (0.25 and 0.29 respectively) than following the injection of radioiodinated growth hormone (1.26 and 0.57 respectively).

Although there was a large absolute I^{131} concentration in the pancreas

[6] These tissues were examined by Dr. Stephen S. Sternberg, assistant attending pathologist, Memorial Center.

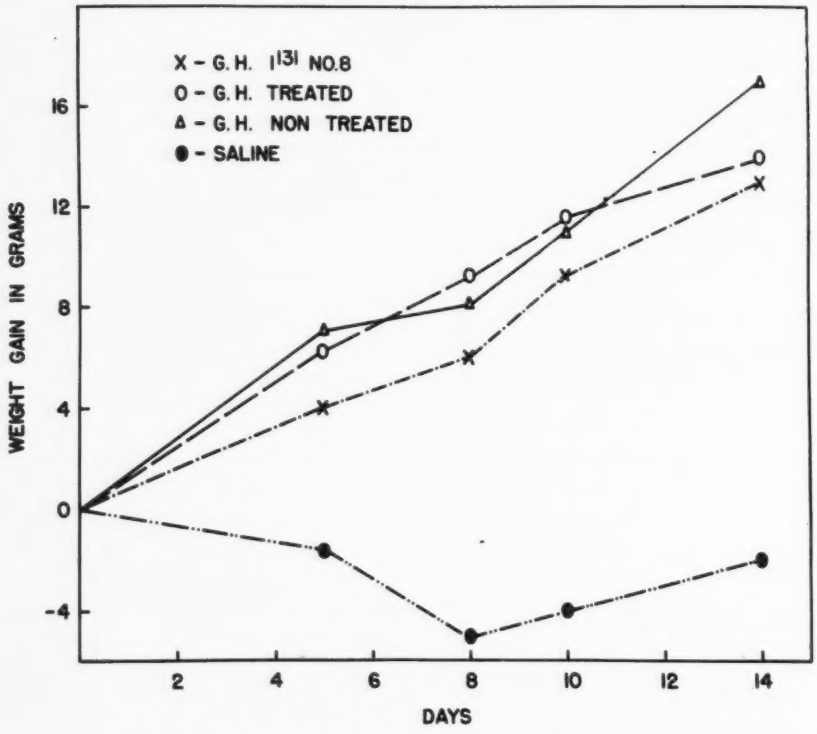

FIG. 1. Biological activity of various growth hormone preparations as assayed by the weight gain of hypophysectomized female rats. G.H. I^{131} No. 8 is a radioiodinated growth hormone preparation. G.H. Treated is a non-radioiodinated growth hormone preparation (See text).

after intracardiac injection as compared to subcutaneous administration of radioiodinated growth hormone, the organ/blood ratios of I^{131} concentrations were not significantly different (Table 2). There were no differences between the sexes as to radioiodine concentration in the pancreas after the injection of radioactive growth hormone to intact rats.

Hypophysectomy at varying periods of time prior to the administration of radioactive growth hormone did not show conclusive differences in the degree of localization of radioactivity in the pancreas (Table 3).

Alloxan diabetic rats demonstrated no significant differences from normal rats in the localization of radioiodine in the pancreas after the administration of radioiodinated growth hormone (Table 4), although pancreases from these animals showed the characteristic beta cell degeneration. Animals pretreated with ethionine showed the characteristic destruction of the

TABLE 1. LOCALIZATION OF RADIOACTIVITY AT DIFFERENT TIMES AFTER THE ADMINISTRATION OF A LABELED GROWTH HORMONE PREPARATION (G.H. I^{131} #1)

Time in minutes	Pancreas		Adrenal		Ovary		Muscle	
	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood
3	0.84	0.22	1.76	0.47	1.70	0.58	0.26	0.07
10	1.68	0.53	3.86	1.22	2.83	0.89	0.50	0.16
60	2.35	2.4	0.92	0.80			0.85	0.74
240	1.73	0.18	1.14	0.78	1.00	0.69	1.08	0.74
1440	0.29	0.61	0.10	0.21	0.37	0.79	0.22	0.46

Time in minutes	Liver		Spleen		Thyroid		Kidney	
	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood
3	4.13	1.10	1.77	0.47	2.63	0.70	13.67	3.64
10	5.60	1.77	2.31	0.73	6.97	2.20	9.58	3.03
60	1.35	1.17	1.24	1.08	20.60	18.00	4.95	4.30
240	1.73	1.19	1.41	0.97	149.7	102.5	3.75	2.58
1440	0.45	0.94	0.40	0.84	451.0	952.0	0.84	1.76

Time in minutes	Epiphysis		Metaphysis		Thymus		Fat		Blood
	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.	Organ / Blood	I^{131} conc.
3	0.84	0.22	0.81	0.22	0.97	0.26	0.32	0.09	3.77
10	1.28	0.40	1.31	0.41	1.08	0.34	0.57	0.18	3.17
60	1.00	0.87	0.84	0.73	1.58	1.37	0.64	0.55	1.15
240	1.16	0.80	1.13	0.77	2.38	1.64	0.57	0.39	0.89
1440	0.40	0.83	0.31	0.65	0.36	0.76	0.09	0.18	0.48

I^{131} concentration refers to the fraction of the administered dose of radioiodine which localized per gram of tissue. The organ/blood ratio is a relationship between the concentration of radioiodine in tissue to the concentration in blood. Each recorded value represents the average determination from two or more animals.

TABLE 2. THE EFFECT OF ROUTE OF ADMINISTRATION OF A LABELED GROWTH HORMONE PREPARATION (G.H. I^{131} #14) ON THE LOCALIZATION OF RADIOACTIVITY IN THE PANCREAS AT VARIOUS TIMES AFTER ITS INJECTION

Route	Time (hours)	I^{131} conc. pancreas	Organ / Blood
Subcutaneous	1	0.096	0.307
	3	0.077	0.229
	5	0.071	0.196
Intracardiac	1	0.203	0.239

I^{131} concentration refers to the fraction of the administered dose of radioiodine which localized per gram of tissue. The organ/blood ratio is a relationship between the concentration of radioiodine in tissue to the concentration in blood. Each recorded value represents the average determination from two or more animals.

TABLE 3. EFFECT OF HYPOPHYSECTOMY ON THE LOCALIZATION OF RADIOACTIVITY IN THE PANCREAS 1 HOUR AFTER THE ADMINISTRATION OF A RADIOIODINATED GROWTH HORMONE PREPARATION (G.H. I^{131} #12) AND I^{131} LABELED INORGANIC IODIDE (I^{131} #11)

Days after hypophysectomy	I^{131} conc.	Organ / Blood
	A. G.H. I^{131} #12	
Intact	2.15	1.32
2	2.57	0.76
4	1.61	0.47
6	1.81	0.60
10	3.64	1.27
	B. I^{131} #11	
Intact	1.25	0.48
2	0.91	0.35
4	0.87	0.32
6	0.61	0.26
8	0.57	0.27
10	0.74	0.39

I^{131} concentration refers to the fraction of the administered dose of radioiodine which localized per gram of tissue. The organ/blood ratio is a relationship between the concentration of radioiodine in tissue to the concentration in blood. Each recorded value represents the average determination from two or more animals.

TABLE 4. EFFECT OF ALLOXAN TREATMENT AND ETHIONINE TREATMENT ON THE LOCALIZATION OF RADIOACTIVITY IN THE PANCREAS 1 HOUR AFTER THE ADMINISTRATION OF A RADIOIODINATED GROWTH HORMONE PREPARATION (G.H. I^{131} #11)

Time (minutes)	I^{131} conc.	Organ / Blood
A. Alloxan Treated Rats (Average Weight at time of Sacrifice—119 gms.)		
3	0.17	0.19
10	0.23	0.52
30	0.28	0.63
100	0.18	0.51
300	0.23	0.89
B. Ethionine Treated Rats (Average Weight at time of Sacrifice—64 gms.)		
3	0.99	0.73
10	0.60	1.46
30	0.69	0.73
100	0.73	1.57
300	0.46	0.62
C. Untreated Rats (Average Weight at time of Sacrifice—156 gms.)		
3	0.17	0.63
10	0.22	0.40
30	0.18	0.62
100	0.14	0.62
300	0.12	0.74

I^{131} concentration refers to the fraction of the administered dose of radioiodine which localized per gram of tissue. The organ/blood ratio is a relationship between the concentration of radioiodine in tissue to the concentration in blood. Each recorded value represents the average determination from two or more animals.

pancreatic acini. When such animals were injected with radioactive growth hormone and comparisons made with normal animals similarly injected, it was found that the pancreatic I^{131} concentration was greater in the case of the ethionine treated animals (Table 4). Although all rats were approximately the same weight at the onset of the experiment, there was a slower weight gain after treatment with alloxan and an absolute weight loss after injections of ethionine.

In the adrenal the greatest concentration of radioactivity was noted 10 minutes after intracardiac injection of labeled growth hormone in contrast to the pancreatic maximum at 1 hour (Table 1, Fig. 2). The concentration of radioactivity in the adrenal fell at a more rapid rate than the concentration of I^{131} in the pancreas. The concentration of I^{131} in the thyroid showed a rapid increase to a maximum at the last point observed, 24 hours (Table 1). All other organs showed significant decreases in radioactivity at 24 hours (Table 1). There appeared to be no significant difference in the localization of radioactivity in the epiphyseal portion of bone in contrast to the metaphysis following the administration of I^{131} labeled growth hormone (Table 1). In many studies performed with male rats, no significant

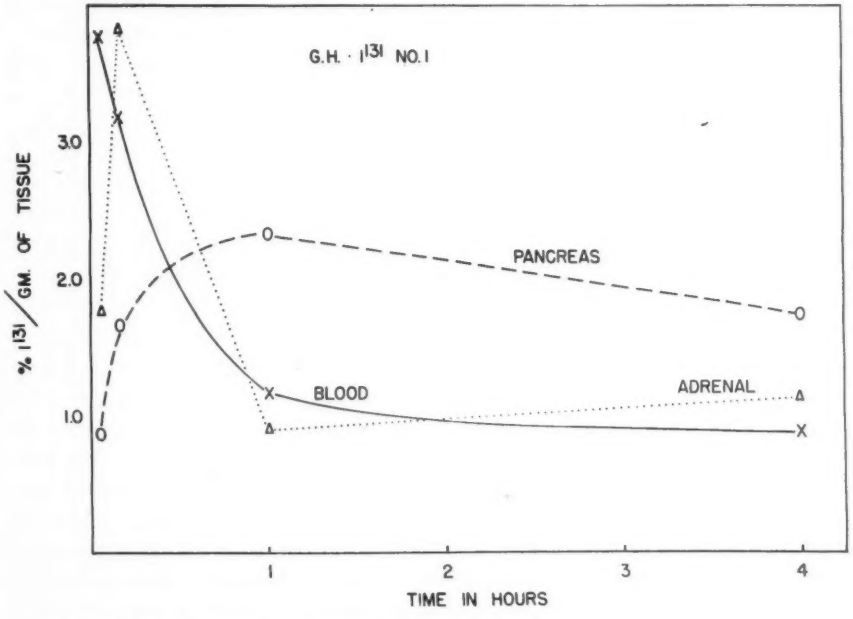

FIG. 2. Relationship of time and concentration of radioactivity in the pancreas, adrenal and blood after the administration of a labeled growth hormone preparation.

localization of radioactivity was noted in the primary or secondary sexual organs after administration of radioactive growth hormone.

DISCUSSION

It has been found (Li, Simpson and Evans, 1948) that after iodination of growth hormone at pH 5.25, iodine to protein molecular ratios of over 4 to 1 have been obtained and these preparations showed only a slight loss in biological activity. In the present study, when radioiodinated growth hormone was prepared with an iodine to protein molecular ratio of 3.5 to 1, most of the biological activity was retained. However, the determination of the biological activity of a substituted protein is restricted by several considerations (Sonenberg, 1952).

In the present study no attempt was made to satisfy all the criteria (Sonenberg, 1952) for specificity of localized labeled components for a tissue, but the radioactivity which localized in the pancreas after the administration of radioiodinated growth hormone satisfied some of these criteria. When the concentration of radioactivity in the pancreas after administration of radioiodinated growth hormone was contrasted with other I^{131} labeled substances (Fig. 3), the greatest localization of radioactivity was noted in the case of labeled growth hormone. Secondly, after the administration of I^{131}-labeled growth hormone, there was a greater concentration of radioactivity in the pancreas than in so-called nonspecific tissues, *e.g.*, muscle and fat. Thirdly, concentrations of radioactivity greater than blood were noted in the pancreas after injecting radioactive growth hormone.

The radioactivity which localized in the pancreas after administration of I^{131}-labeled growth hormone is probably associated with the protein and is not the result of growth hormone biological activity which promotes I^{131} accumulation after the radioiodine is free of the protein. This conclusion is drawn from the observation when I^{131}-labeled inorganic iodine was administered with non-radioactive growth hormone preparations, there was not the same degree of localization of radioactivity in the pancreas as was noted after the injection of radioiodine labeled growth hormone.

Some insight into the histological site of localization of radioactivity, in the pancreas may have been obtained from the distribution studies in pretreated animals. The administration of ethionine results in the destruction of pancreatic acinar tissue (Farber and Popper, 1950; Goldberg, Chaikoff and Dodge, 1950) and has been realized in our experiments. We observed a greater localization of radioactivity after the administration of radioactive growth hormone in the pancreases of ethionine treated rats than in control animals. This is consistent with the concept that there was a rela-

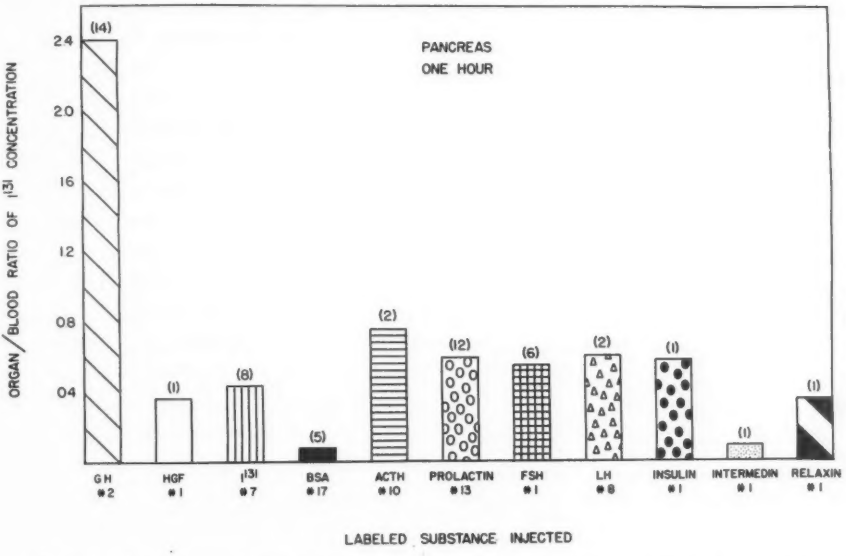

FIG. 3. The relationship of I^{131} concentration between pancreas and blood one hour after the administration of different labeled substances.

GH—Growth Hormone; HGF—Hyperglycemic Factor; I^{131}—radioactive iodine; BSA—Bovine Serum Albumin; ACTH—Adrenocorticotrophin; FSH—Follicle Stimulating Horome; LH—Luteinizing Hormone.

Numbers in brackets above columns represent the number of preparations of each labeled substance studied. Each animal received 100 micrograms of protein except those receiving carrier-free amounts of radioactive inorganic iodide.

GH	(Armour 22KR2)	LH	(Armour SL-264-107B)
HGF	(Horner 208-108B-234)	FSH	(Parke Davis R_x 124541)
BSA	(Armour J4902)	Insulin	(Armour 7-5)
ACTH	(Armour J9701)	Intermedin	(Armour MH-423-7z plus 489A-1)
Prolactin	(Squibb 71713)	Relaxin	(Chilcott 6-6-65-S)

tive increase in islet tissue after the ablation of acinar tissue and the localized radioactivity in the pancreas was associated with the islet cells. Some caution must be employed in interpreting these data in view of the generalized toxic effect of ethionine on the organism as a whole. However, when the beta cells of the pancreas were destroyed with alloxan, there was no significant difference in the concentration of radioactivity in the pancreas after injecting I^{131}-labeled growth hormone to normal and alloxanized animals. It would appear, then, that the radioactivity may be associated with the alpha cells, delta cells, or some other element in the pancreatic islets. However, without any radioautographs, one can not be certain that the radioactivity is indeed associated with the islet tissue. In addition, the

specificity of this increased localization of radioactivity after treatment with ethionine will have to be established by administering other radioiodinated substances to similarly treated rats.

Hypophysectomy does not alter the property of the pancreas to concentrate radioactivity when administered as I^{131}-labeled growth hormone. This seemed to be the case whether the hypophysis was removed from 2 to 10 days prior to the administration of the labeled growth hormone. If the experimental animal's own production of growth hormone would dilute the amount of labeled growth hormone which localized in the pancreas, one would anticipate that this effect would be minimal ten days after hypophysectomy. At this time the bulk of the endogenous growth hormone in blood should be absent if we can employ the biological half life of growth hormone as being approximately 26 minutes (Li, 1950–51; van Dyke, Simpson, Li and Evans, 1950). Perhaps the administration of 100 μg. of growth hormone far exceeded the normal quantity of this hormone present in blood. If so, the blood concentration of growth hormone would be similar in intact as well as in the experimental animals. No data are available as yet with the administration of lesser amounts of growth hormone. It is also possible that the biological half life in blood may be significantly prolonged in the hypophysectomized animals or the renal or extrarenal clearance of this material is markedly reduced. Under such circumstances there may be no significant alteration in the pancreatic localization of radioactivity after the administration of radioactive growth hormone.

The localization of radioactivity in the adrenal after injecting radioiodinated growth hormone may reflect contamination of this growth hormone preparation with adrenocorticotrophin, despite the negative biological assay, or reflect the levels of radioactivity in blood. It should be noted, however, that the radioactivity in the adrenal reached its maximum ten minutes after injection while the administration of I^{131}-labeled ACTH preparations resulted in maximal adrenal concentration in 30 seconds (Sonenberg, Keston, and Money, 1951). After the administration of I^{131}-labeled growth hormone, the concentration fell from a maximal value at 10 minutes with a half life greater than 5 to 6 minutes, while the net half life of radioactivity from the adrenal with labeled ACTH preparations was approximately 5.5 minutes. In studies with labeled growth hormone, no attempt has been made to partition the radioactivity between cortical and medullary portions of the adrenal by radioautography.

With all of our labeled anterior pituitary preparations (Sonenberg *et al.*, 1951, 1952), significant localization of radioactivity has been noted in the liver and spleen. This has been attributed to the action of the reticuloendothelial system on a foreign protein. In the experiments with labeled

growth hormone, it has not been excluded that there may be a specific localization in liver of some component in this preparation.

Although the localization of radioactivity in the thymus may be specific, the technique of intracardiac injection permits contamination of thymic tissue with radioactive materials. Further experiments will be necessary to establish the specificity of radioactivity in the thymus of animals that have received radioactive growth hormone.

The localization of radioactivity in the thyroid has been interpreted to reflect the uptake of inorganic I^{131} by this tissue after the I^{131}-labeled proteins have been degraded. This is suggested by the greater uptakes at the later periods of time after injection. It has been observed that this localization is markedly reduced in hypophysectomized animals. The large localization of radioiodine in the kidney, like the large uptake by the thyroid is consistent with the notion that inorganic radioiodine is being excreted after the labeled protein has been degraded.

These experiments in no way establish the epiphyses as a specific site of localization for radioactive growth hormone. It should be noted that the concentration in the epiphyses was not significantly greater than that noted in the metaphyses. Experimentally, only the epiphyseal end of the bone was examined and no attempt was made to isolate the epiphyseal plate. Perhaps this resulted in some dilution at the precise site of localization. In preliminary studies, radioautographs of the epiphyseal area demonstrated most of the radioactivity to be at the epiphyseal line of rats receiving I^{131}-labeled growth hormone. However, I^{131}-labeled iodide and radioiodinated serum albumin gave similar radioautographs.

SUMMARY

An anterior pituitary preparation with growth hormone activity has been labeled with radioactive iodine. The biological activity of this labeled preparation has been retained. Significant localization of radioactivity has been noted in the pancreas, adrenal, liver, spleen, thyroid, kidney and thymus after administering labeled growth hormone preparations to the rat. The localization of radioactivity in the pancreas would appear to predominate in the islets, but not in the beta cells. The specificity of radioactivity in the epiphyses after injecting this labeled preparation has not been established with the data now available.

Acknowledgment

The authors would like to express their appreciation to Dr. Rulon W. Rawson for his advice and encouragement during these studies.

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THE MICRO ESTIMATION OF STEROIDS PRODUCED BY RAT ADRENALS *IN VITRO*[1]

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RAT adrenal glands have been found by Saffran, Grad, and Bayliss (1952) to produce steroids when incubated in a saline medium and to be stimulated to form much larger amounts when ACTH is added to the medium. Others have shown that slices of pig and beef adrenal cortex (Haynes, Savard, and Dorfman, 1952) can be similarly stimulated by ACTH. The methods used in these latter studies for extraction and purification of the steroidal material prior to estimation have involved much manipulation of the samples with attendant losses of material. The methods described here are rapid and quantitative. The ultraviolet absorption method for measuring the steroids, used by Saffran *et al.* (1952) and Saffran and Bayliss (1953) has been improved and adapted to the measurement of 2 to 5 μg. of steroid with an error of less than ±10%. A second method, making use of a derivative of triphenyl tetrazolium chloride, has been developed to measure the steroids in the same low amounts and with the same accuracy. Both methods can be used to measure the *in vitro* steroid production of a single rat adrenal.

A comparison of the steroid values of adrenal incubation media obtained by these two methods is of interest since the ultraviolet method (UV) measures steroids containing the Δ^4-C-3 ketone group and the tetrazolium chloride derivative (M. & B. 1767), steroids with an α-ketol side chain. Data are presented showing that consistently higher values are obtained by the UV method than by the chemical method in the presence and absence of added ACTH. This indicates that in addition to the known corticoids the isolated rat adrenal produces substances with an α,β-unsaturated ketone group but without the characteristic α-ketol reducing side chain. The formation of both kinds of substances is stimulated by added ACTH.

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METHODS

Incubation of the glands

One to 5 bisected rat adrenal glands were incubated in either Krebs-Ringer-0.025 M bicarbonate and 95% oxygen-5% CO_2 or in Krebs-Ringer 0.01 M phosphate and 100% O_2. The glucose concentration was 200 mg. %. Care was taken to prevent the precipitation of calcium in the medium because calcium is necessary for the stimulation of the rat adrenal by added ACTH (Birmingham, Elliott and Valère, 1953). The glands were incubated without ACTH for 1 hour at 37° C. prior to the experimental period (Saffran and Bayliss, 1953). The medium, referred to later as preincubation medium, was then removed and incubation in fresh medium with or without added ACTH (0.02 to 0.1 unit/ml.) was continued for 2 hours under the same conditions. To reduce animal variation the glands were paired as previously described (Saffran and Bayliss, 1953).

An aliquot of the incubation medium was removed to a glass stoppered test tube.[3] An accurately measured volume of chloroform was added (about 2.5 times the volume of the aliquot taken). The tube was shaken 100 times, then centrifuged 5-10 minutes at 3,500 RPM. As much as possible of the chloroform solution was delivered onto a V-shaped piece of chloroform-extracted filter paper placed in the neck of a centrifuge tube. Droplets of water carried with the chloroform are caught by the paper. An aliquot of the chloroform solution was transferred to a small test tube and placed in an evaporation apparatus to remove the solvent.

The evaporation apparatus consists of an evaporation chamber made from a 25 ml. test tube with a ground glass joint through the top of which a sealed-in glass tube passes and reaches to within 3 to 5 cm. of the bottom of the chamber. The chamber has also a sealed-in side arm. The small test tube containing the chloroform solution was placed in the chamber which was kept at 55° C. in a water bath. A slow stream of nitrogen delivered through the vertical tube prevents bumping of the chloroform while it is evaporated by a water pump connected through the side arm. Evacuation for 30 minutes with a rotary oil pump removes the last traces of chloroform. The entering nitrogen passes through a drying column and glass wool trap. Removal of the oxygen contamination is unnecessary (Nelson and Samuels, 1952).

Measurement of the steriods by ultraviolet absorption

To the residue from the incubation medium of 1 to 3 glands, 0.5 ml. of methyl alcohol was added. The tubes were stoppered, shaken gently and allowed to stand for 30 minutes to dissolve the steroids completely. The solution was placed in a small silica cuvette (light path, 10 mm.; capacity, 0.3 ml.) and its optical density measured in the Beckman DU spectrophotometer at wave lengths, 225, 240, 255 and 260 mμ. against a methanol blank. An aliquot of the medium used was extracted in the same way as the incubation medium and a methanol solution of this residue was used as a blank control.

Precautions necessary for accurate UV absorption measurements

Special care must be taken to prevent the introduction of impurities, that absorb ultraviolet light, in every step in the incubation, extraction and evaporation procedures. The methanol and chloroform must be redistilled before use. All glassware must be cleaned

[3] Two ml. volumetric test tubes are suitable for the extraction of 0.4 to 0.75 ml. aliquots of medium; 9 ml. glass stoppered test tubes are useful for 1 to 2 ml. aliquots; 25 ml. test tubes, previously described (Saffran, Grad, and Bayliss, 1952), are suitable for larger amounts.

especially carefully and kept in an enclosed space if not used immediately after drying. The filter paper triangles, made from Whatman #40 paper, should be extracted with chloroform in a Soxhlet apparatus for 2 to 3 hours and stored in a closed space until used. All the tubes and passage ways in the evaporation apparatus must be clean so that particles are not carried over into the sample flask during pressure changes. A manometer in the system is useful especially at the end of the evacuation to ensure a slow entrance of air into the apparatus. If solvent fumes are present in the atmosphere, it is necessary to fill the evaporation apparatus with nitrogen.

Calculation of steroid values from their UV absorption curves

Since many substances absorb ultraviolet light in the wave length region in which steroids have their maximum absorption, 240 mμ., it is difficult to measure steroids accurately by this method. When it can be assumed that the impurities have a straight line absorption curve in the 240 mμ. region, the value of the substances to be measured can be calculated from the relationship of the optical density (*OD*) at 240 mμ. to the *OD*s at two other wave lengths, one at a longer and the other at a shorter wave length (Allen, 1950). Such a calculation has been used by Saffran, Grad, and Bayliss (1952) for steroid determinations: calculated density, $CD_1 = OD_{240} - \frac{1}{2}(OD_{225} + OD_{255})$. If the absorption curve of the impurities has a reasonably long horizontal section in the 240 mμ. wave length region, the *OD* of the unknown at 240 mμ. can be compared with the *OD* at another wave length in this horizontal section, such as, calculated density, $CD_2 = OD_{240} - OD_{260}$. When the interfering substances have an absorption curve that is convex to the wave length axis, neither calculation would give the correct value. However, by considering known and probable variations in the absorption curve of impurities encountered and how they would affect calculated densities chosen, such as CD_1 and CD_2, it is possible to justify using the difference between the values obtained as a measure of the error that the interference causes.

It has been found that a methanol solution of the dried chloroform extract of medium alone has a convex absorption curve, such as curves *B* in Figure 1A and B. A similar absorption curve has been obtained for a methanol solution of dried extract of medium in which adrenals were incubated at 0° C. or in an atmosphere of nitrogen for two hours (Saffran and Bayliss, 1953). Examination of the absorption curves of the solutions of many extractions of medium alone show that the main differences among the curves are found in the 225 mμ. region, where the *OD* is often very high, while the curves in the 240–260 mμ. region are either horizontal or nearly so.

In Figure 1A, curve *U* is the absorption curve of a solution containing interfering material, the absorption curve of which is curve *B*, and an amount of steroid, which, without contamination, would give curve *S*. The CD_1 values of curves *U*, *S*, and *B* are respectively *u*, *s*, and *b*, by definition. The CD_1 value of *U* is equal to *s* minus *b*, since curve *U* is the composite of curves *S* and *B*. Thus the steroid value of curve *U*, calculated by its CD_1 value, *u*, will be in error and too low by an amount, *b*.

In Figure 1B, the $OD - OD_{260}$ values of curves *U*, *S*, and *B* have been plotted and their respective CD_2 values, *u'*, *s'*, and *b'*, are shown. If one uses *u'* as a measure of the steroid having curve *U*, it will give too high a value for the steroid by *b'* since $u' = s' + b'$.

If the curve for interfering material, *B*, had decreased linearly with increasing wave length, *u* would equal *s* (Fig. 1A) but *u'* would still be greater than *s'* (Fig. 1B).

If there is no interfering material, $u = s$ and $u' = s'$, and the steroid value obtained from *u* will equal the steroid value obtained from *u'*. Thus if the steroid value for a solution giving curve *U* is calculated by the two methods, from CD_1 and CD_2, and there is no dif-

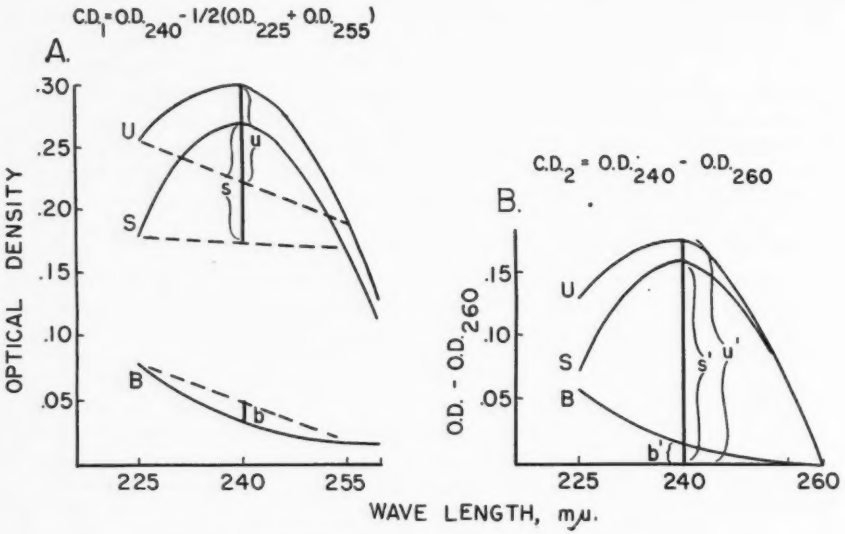

FIG. 1. Graphical Representation of CD_1 and CD_2.

S = absorption curve of pure steroid;

B = absorption curve of interfering substance;

U = absorption curve of unknown containing interfering substance;

s and s' = calculated optical densities (CD_1 and CD_2) of pure steroid derived by equations given in Figs. 1A and 1B respectively;

b and b' = CD_1 and CD_2 of interfering substance;

u and u' = CD_1 and CD_2 of unknown containing interfering substance.

ference in the value, it is reasonable to assume that no interfering material is present to distort the steroid curve S. The difference between the values calculated by CD_1 and CD_2 is a mathematical expression of the distortion of the steroid curve and gives an estimate of the interference. Moreover, the true steroid value must lie within the limits of these two calculated values.

In Table 1 are given the average differences between steroid values obtained from calculations using CD_1 and CD_2 for methanol solutions of chloroform extracts of medium alone and medium in which adrenals have been incubated. The average impurities are shown to be much greater and significantly different for each medium before precautions described above were taken as compared to the impurities after precautions were taken. The data indicate further that the amount of impurities present in extracts of incubation media (line 5) are on the average no greater than the amount of impurities extracted from medium alone (line 6) and therefore the source of impurities is not the adrenal gland. However, since the amount of impurities extracted from preincubation media (line 4) is larger than that from medium alone (line 6), these extracts probably contain impurities of adrenal origin. It might be inferred that the steroid value for incubation media could be corrected by subtracting the blank value for an extract of medium alone. However, when a blank extraction determination is carried out in conjunction with a series of experimental extractions, the blank value does not always correspond to the

TABLE 1. DIFFERENCES IN STEROID VALUES OBTAINED FROM UV ABSORPTION CURVES BY CALCULATION FROM CD_1* AND CD_2* FOR EXTRACTS OF MEDIA

	No. of tests	Mean CD_1, CD_2 difference ±S.E.
Before Precautions described were taken		
1. Preincubation media	20	3.40 ± 0.538
2. Incubation media	49	2.28 ± 0.213
3. Krebs-Ringer media	20	1.94 ± 0.378
After Precautions described were taken		
4. Preincubation media	26	1.41 ± 0.112
5. Incubation media	135	0.97 ± 0.054
6. Krebs-Ringer media	50	0.94 ± 0.063

	Probability that the difference is due to chance (Snedecor, 1948)
Pair	
1 vs 4	$0.01 > p > 0.001$
2 vs 5	$p < 0.001$
3 vs 6	$0.01 > p > 0.001$
4 vs 5	$p < 0.001$
5 vs 6	$p > 0.9$

* See text and Figure 1 for definitions of CD_1 and CD_2.

amount of impurity calculated by CD_1 and CD_2 for each experimental extract.

The error due to impurities in incubation media has been shown in Table 1 (line 5) to average ±0.5 μg. steroid/ml. methanol. In order to obtain steroid values with an error no greater than ±10% a concentration of 5 μg. steroid/ml. methanol must be measured. This concentration can be measured in a microcuvette with only 1.5 μg. steroid in 0.3 ml. methanol.

The factors obtained for converting the CD_1 and CD_2 values to μg. steroid per ml. are listed in Table 2. When a sample contained a mixture of steroids, the factors for 17-hydroxycorticosterone (compound F) were used. Only in the unlikely case of the presence of a large amount of desoxycorticosterone (DOC) would the use of these factors introduce a significant error.

Measurement of steroids by M. & B. 1767

To measure the α-ketol side chain of steroids a derivative of triphenyl tetrazolium chloride, 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (M. & B. 1767), recommended to us by Dr. Henley (cf. 1951 and 1952b), has been used. The procedure for its use has been adapted from that described by Mader and Buck (1952) for 3,3-dianisole-bis-4,4′-(3,5-diphenyl) tetrazolium chloride. The procedure used by Dr. Henley (1952a) for M. & B. 1767 includes a final neutralization which we have omitted.

In studying the effects of conditions on the reduction of M. & B. 1767 by compound

TABLE 2. FACTORS FOR CONVERTING CD_1* AND CD_2* VALUES TO μG. STEROID PER ML.

	DOC	Cpd. B	Cpd. E	Cpd. F
CD_1	44	53	56	55
CD_2	27.5	33	31	34

* See text and Figure 1 for definitions of CD_1 and CD_2.

F, 11-dehydro-17-hydroxycorticosterone (compound E), corticosterone (compound B), and DOC it was found that at a reaction temperature of 28° C. a reasonably intense yellow red color develops (optical density of 0.100 = 1 μg. steroid). The color is quite stable for about 30 minutes. At higher reaction temperatures a more intense but also more unstable color forms. The factors for converting the optical densities at 510 mμ. of compounds F, E, and B and of DOC to μg. steroid/ml. reaction mixture have been found to be 22, 27, 22, and 19 respectively. Progesterone and androsterone cause no reduction of M. & B. 1767 under the same conditions nor do residues of chloroform extracts of Ringer-Krebs medium containing glucose. There is some variation in the conversion factors given above so that standard solutions of a known steroid must be used as controls with each series of determinations.

Reagents:

1. A solution of 1.5 mg. of 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (M. & B. 1767) per ml. of 95% ethyl alcohol. This solution must be prepared fresh daily.
2. A solution of tetramethylammonium hydroxide in ethyl alcohol. Three-tenths ml. of a 10% aqueous solution of the hydroxide is diluted to 10 ml. with 95% ethyl alcohol.

An aliquot of the methanol solution of extracted steroids, or preferably an aliquot of the chloroform extract of steroids, is evaporated in the apparatus described above. To the residue is added 0.2 ml. of redistilled 95% ethyl alcohol. The sample is kept stoppered for 30 minutes in a water bath at 38° C. to assure complete solution of the residue. Then 0.15 ml. of reagent *2* is added, followed immediately by 0.15 ml. of reagent *1*. The contents are mixed and the tube is placed in a water bath at 28° C., protected from light, removed after 20 minutes and measured within 20 minutes in the Beckman spectrophotometer in microcuvettes at 510 mμ.

RESULTS AND DISCUSSION

Recovery experiments

In Table 3 are shown the results of experiments in which compounds F, E, and B and DOC were added to Ringer-Krebs medium, extracted and measured by ultraviolet absorption (UV method) and by reduction of M. & B. 1767 (MB method). They were added in amounts such that their concentration per ml. medium was 5 to 22 μg. These concentrations are within the range found in adrenal incubation media under the conditions described, 2 to 40 μg./ml. medium.

The recoveries from Ringer-Krebs medium alone (Table 3, A) show that the extraction and purification procedures involve no losses of steroids, even though there is only one chloroform extraction. The agreement between the recoveries calculated from CD_1 and CD_2 indicates that there were practically no impurities present to interfere in the UV measurements. Good recoveries were also obtained as estimated by the MB method and agreed with those obtained by the UV method where both determinations were made on the same extracts.

Equally satisfactory recoveries of known steroids added to tissue incu-

TABLE 3. RECOVERY OF STEROIDS

No. of tests	Steroids added	Amount for measurement μg.	UV method		*MB* method Avg. %
			Avg. % using CD_1*	Avg. % using CD_2*	
A. Added to Ringer-Krebs Medium					
11	DOC	1 to 2	95 (80–108)	100 (88–119)	—
11	DOC	2 to 5	95 (82–104)	99 (91–121)	—
2	F, E, B	13	97 (96–97)	98 (97–99)	—
1	F, E, B	4.5	91	99	100
2	F	4	—	—	93 (93–93)
B. Added to Ringer-Krebs Medium in which Tissue had been Incubated 2 Hours					
			Adrenal		
1	F, E, B	25	105	98	—
6	F, E, B	7.5	101 (94–108)	99 (89–106)	100 (94–107)
			Heart		
6	F	4	—	—	91 (87–95)
			Liver		
4	F	4	—	—	84 (74–93)

The values in parentheses are the highest and lowest percentages of the series of values averaged.

* See text and Figure 1 for definitions of CD_1 and CD_2.

bation media were obtained (Table 3, B). They indicate that the chloroform-extractable material from tissue incubation media does not interfere in the estimation of steroids by either method.

Steroid values of adrenal incubates

In Table 4 are shown the analytical data from many incubation experiments carried out as described. The average steroid values obtained by the two methods are shown for each of the three types of incubation media analyzed. The stimulatory effect of ACTH was calculated from the difference

TABLE 4. STEROID VALUES OF ADRENAL INCUBATION MEDIA MEASURED BY THE UV AND MB METHODS

	Preincubation media	No added ACTH	With added ACTH	Calculated effect of ACTH
		μg./100 mg. adrenals		
MB method				
No. tests	(9)	(10)	(18)	(10)
Mean	8 ± 3.2 (S.D.)	7 ± 1.3	19 ± 5.2	11 ± 5.0
UV method				
No. tests	(9)	(10)	(18)	(10)
Mean	15 ± 5.5	14 ± 3.7	32 ± 7.7	17 ± 8.5
MB/UV ratio				
No. tests	(9)	(10)	(18)	(10)
Mean	0.52 ± 0.05	0.53 ± 0.03	0.61 ± 0.06	0.73 ± 0.11

TABLE 5. EFFECT OF ACTH ON THE MB/UV RATIOS OBTAINED WITH PAIRED ADRENAL GLANDS

No added ACTH	With added ACTH	Calculated effect of ACTH
0.50	0.63	0.83
0.50	0.62	0.75
0.66	0.62	0.60
0.55	0.69	0.78
0.50	0.59	0.73
0.43	0.59	0.78
0.42	0.50	0.58
0.74	0.64	0.60
0.50	0.67	0.92
0.50	0.61	0.71

The differences between the paired values in Columns 1 and 2, analyzed by Student's *t*-test (Student, 1925) are significant at the 0.02 level; those between Columns 1 and 3, at the 0.01 level.

in the amount of steroids produced by the glands incubated with and without added ACTH. The glands in the presence of added ACTH produced 1.5 to 4 times as much steroid as the glands incubated without ACTH, with which they could be compared. The same results have been obtained in many other experiments carried out in the same way in which the steroids formed were measured by the UV method only. The failure of Hofmann (1954) to obtain a consistent stimulating effect of ACTH on the steroid production of rat adrenals *in vitro* is, we believe, due to the lack of calcium in his media (Birmingham *et al.*, 1953). The ratio of the steroid value obtained by the MB method to that obtained by the UV method for every medium analyzed has been invariably less than 1 indicating that the two methods are not measuring entirely the same steroids.

Table 5 lists the MB/UV ratios obtained after incubation of paired adrenal glands with and without added ACTH. The differences in the ratios in each pair are statistically significant indicating that ACTH increased the proportion of material measured by the MB method.

Of the steroids identified in adrenal cortical extracts, only the six corticoids and aldosterone would give the same value by the two methods used, i.e., they are the only steroids with the α-ketol side chain at C_{17} (measurable by M. & B. 1767) and also the Δ^4-3-ketone group (measurable by UV absorption). If the adrenal glands, when incubated, had formed only these corticoids, the MB/UV ratios would have been 1.0. The discrepancy in the steroid values obtained by these two methods could be explained if rat adrenals formed a steroid of the type found by Brady (1953) in incubation media of dog adrenal slices, namely, a compound with an $\alpha\beta$-unsaturated ketone group but without a reducing ketol side chain and with a polarity like that of Compound E. Bush (1953) has obtained a component from the adrenal vein blood of rats which he believes to be 11-hydroxyandrost-

4-ene-3, 17-dione and which has a polarity less than that of corticosterone. We have chromatographic evidence, Elliot and Shally, 1955, that the component(s), which mainly accounts for the discrepancy discussed, has a polarity similar to the compound described by Brady.

Although ACTH stimulates mainly steroids measured by the MB method, probably corticoids (Table 5), it also stimulates the formation of steroid(s) without the α-ketol side chain, since the MB/UV ratio of the calculated effect of ACTH is also less than 1 (Table 4).

SUMMARY

Two procedures are described which permit a rapid estimation of as little as 2 μg. of corticoids. By these methods the *in vitro* steroid production of a single rat adrenal can be measured with an error of $\pm 10\%$.

Steroids with a Δ^4 C-3 ketone group are measured by ultraviolet absorption and α-ketol steroids, by 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (M. & B. 1767).

It was found from analysis of media in which rat adrenals had been incubated with and without added ACTH that more ultraviolet absorbing material is formed than material with an α-ketol group and that ACTH stimulates the formation of both types of material. The ratio of the α-ketol material to ultraviolet absorbing material is greater for incubation media of glands stimulated by ACTH than for incubation media of unstimulated glands. The significance of these findings has been discussed.

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RESPONSE OF THE HAMSTERS' ADRENAL CORTEX TO DESOXYCORTICOSTERONE ACETATE, WITH OBSERVATIONS ON THE KIDNEY, THYROID AND HYPOPHYSIS

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THE administration of small doses of desoxycorticosterone acetate (DCA) does not affect significantly the weight of the adrenal glands of the rat or mouse (Selye, 1940; Mulinos *et al.*, 1942; Leathem, 1951; Miller, 1953), although the zona glomerulosa of the rats' adrenal may exhibit a decrease in the size of its constituent cells, a loss of sudanophilic lipid, and other signs of "compensatory atrophy" (Greep and Deane, 1947; Zweifach *et al*, 1951). A similar, selective response of the zona glomerulosa is reported to occur when rats are placed on sodium-deficient or sodium-rich diets (Deane *et al.*, 1948; Nichols, 1948; Bacchus, 1950). Hypophysectomy of the rat, in contrast, results in a marked involution of the zona fasciculata and only minimal changes in cells of the zona glomerulosa (Deane and Greep, 1946). Because of these apparently specific responses of the zona glomerulosa, it has been suggested that this zone may be concerned with the elaboration of mineralo-corticoids and that its secretory activity is relatively independent of hypophyseal control (Swann, 1940; Greep and Deane, 1947).

When larger doses of DCA are administered, there occurs a reduction in adrenal weight, caused by involution of the zona fasciculata as well as the zona glomerulosa (Selye and Dosne, 1942; Selye and Stone ,1950; Odenthal, 1952). It is postulated that DCA may inhibit the secretion of pituitary corticotropin to produce this atrophy of the zona fasciculata (Sayers and Sayers, 1947).

Conditions which affect the secretion of corticotropin should be followed by changes in the number and cytology of the pituitary cell type responsible for its elaboration. There is, however, no agreement at present as to which cell type is concerned. Halmi and Barker (1952) produced marked adrenal atrophy in groups of male rats treated with 2 and 5 mg. of cortisone for 83 and 20 days respectively, but found no significant alterations in pituitary cytology. Likewise, Baker (1952) was unable to detect changes in the hypophyses of rats given large doses of corticotropin. Tuch-

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mann-Duplessis (1951b), studying the hypophyses of adrenalectomized rats and rats given DCA, cortisone and corticotropin, concluded that the basophil is the cell which secretes corticotropin. Halmi (1951) and Halmi and Bogdanove (1951a, 1951b) believe that the granules in pituitary chromophils do not represent or reflect the content of corticotropin in the rats' hypophysis. Finnerty *et al.* (1949, 1952), using the acid hematein technique for phospholipids, observed a marked increase in the number of intensely staining acidophils in rats subjected to stressful conditions, and believe that this cell is the source of corticotropin.

The current investigation was undertaken to determine whether the zona glomerulosa in another species, the hamster, can be influenced selectively by DCA. The hypophyses of these animals were examined to detect any change suggesting the cell type responsible for secretion of corticotropin. Because the hypophysis secretes a number of hormones, the possibility must be considered that DCA may alter pituitary cytology by influencing the secretion of hormones other than corticotropin. The influence of DCA on the histology of the thyroid gland and the secretion of thyrotropin are of interest in this regard in view of the fact that numerous workers have reported effects of DCA, cortisone, or corticotropin on thyroid activity (Perry, 1951; Money *et al.*, 1951; Migeon *et al.*, 1952; Halmi and Barker, 1952).

MATERIAL AND METHODS

Fifty-six young adult male hamsters, averaging 68 to 72 gm. in initial body weight, were given daily subcutaneous injections of desoxycorticosterone acetate[1] for 10 days. Three different groups of animals received 0.5, 1, and 2 mg. respectively, with control animals receiving appropriate amounts of sesame oil. The voluntary food intake (powdered Rockland rat diet) was measured daily.

At autopsy, the adrenals were freed of fat, weighed, and prepared for histological study by the following methods: (a) fixation in Bouin's fluid and staining by the Masson technique; (b) Regaud's fluid, with staining by iron hematoxylin for mitochondria; (c) fixation in formol-Zenker-osmic acid for osmiophilic lipid; (d) formalin-fixed adrenals were sectioned on the freezing microtome, stained with sudan black B, sudan III, the Schiff reagent, 2-hydroxy-3-naphthoic acid hydrazide (Ashbel and Seligman, 1949), and tested for cholesterol by the Schultz method; (e) adrenals fixed in chilled 80% alcohol were used for the demonstration of alkaline phosphatase (Gomori, 1939) and esterases (Gomori, 1952). Using Bouin-fixed adrenals, the nuclear areas of cells in the zona glomerulosa and zona fasciculata were measured by making camera lucida drawings (×1680) of the nuclei and measuring their areas with a planimeter; details of this procedure have been described more fully elsewhere (Knigge, 1954b). The heart, kidneys, and thymus were weighed and fixed in Bouin's fluid, the kidneys and thymus being sectioned at 5 μ and stained by the Masson technique. Thyroid glands were fixed and stained in a similar fashion. Hypophyses were fixed in Bouin's fluid, sectioned at 4 μ in a coronal plane,

[1] Desoxycorticosterone acetate was generously supplied by Dr. Robert Gaunt of Ciba Pharmaceutical Products, Inc., Summit, N. J.

TABLE 1. THE BODY AND ORGAN WEIGHTS OF MALE HAMSTERS TREATED FOR 10 DAYS WITH DESOXYCORTICOSTERONE ACETATE

Group	No. of hamsters	Body weight, g.		Organ weights, mg.			
		Initial	Final	Adrenals	Kidneys	Heart	Thymus
Control	18	72±1.5	78±1.4	19.4±0.71	824±15	288±11	41.8±4.0
DCA, 0.5 mg.	8	69±1.7	76±2.1	16.8±0.92	1267±13	—	—
DCA, 1 mg.	15	71±1.8	78±5.3	15.4±0.65	1289±36	342±11	41.4±2.3
DCA, 2 mg.	10	68±1.2	74±0.8	13.0±0.34	1280±54	317± 7	43.0±1.4

and stained by the modified (long) Masson technique. Differential counts of hypophyseal cell types were performed by counting the cells in 12 oil immersion fields in 4 sections, following a pattern described by Rasmussen and Herrick (1922). An average of 1728 cells were counted in each hypophysis. Other sections of Bouin-fixed hypophyses were stained by the periodic acid-Schiff technique for polysaccharides as described by McManus (1948).

RESULTS

Body and organ weights. Administration of 0.5, 1, or 2 mg. of DCA for 10 days does not influence body or thymus weight (Table 1). The slight increase in weight of the heart is not significant at the 1% level (Student "t" test). The kidneys at autopsy are larger and somewhat lighter in color than normal, but their surface is not irregular or pitted. Kidney weight is increased approximately 55% by all 3 dosage levels of DCA. The adrenals exhibit a progressive weight loss with increasing dosages of DCA (Table 1), the decrease in weight being significant in all 3 groups. The adrenal weight (13.0 mg.) of animals treated with 2 mg. of DCA for 10 days is slightly greater than that observed in male hamsters hypophysectomized for 30 to 60 days (9.4 mg., compared to 19.8 mg. for littermate controls. Knigge, 1954a).

Adrenal Cortex

Administration of 0.5, 1, or 2 mg. of DCA for 10 days produces a progressively more marked atrophy of the cortex of the adrenal gland, the medulla remaining histologically normal. The histology and histochemistry of the normal hamster adrenal cortex have been described in detail (Knigge, 1954a). Pertinent histology of the normal cortex and changes produced by 2 mg. of DCA will be described here.

Zona glomerulosa. The zona glomerulosa of normal adrenals, as seen in Bouin-fixed, Masson-stained glands, is a narrow, relatively inconspicuous zone which stains lighter than the adjacent zona fasciculata, and which varies regionally in thickness (Fig. 3). The constituent cells, which possess indistinct cell boundaries, are arranged in rounded clusters or are ordered irregularily and not demarcated sharply from the zona fasciculata. Glomerulosal cells contain vacuoles which are preserved poorly after Bouin fixation. The nuclei of glomerulosal cells vary markedly in shape and exhibit a wide range of sizes. The distribution curve of nuclear areas of normal

glomerulosal cells (Fig. 1) is similar to that observed previously (Knigge, 1954b). After fixation in Regaud's fluid and staining with iron hematoxylin, mitochondria of glomerulosal cells are observed as lightly stained, granular bodies which are distributed uniformly in the cytoplasm. The lightly staining mitochondria in glomerulosal cells contrast markedly with those of the zona fasciculata, which are colored intensely (Fig. 13). After fixation in Regaud's fluid, cytoplasmic vacuoles are well preserved. Sudanophilic lipid and cholesterol, as demonstrated after fixation in 10% formalin, are absent from the zona glomerulosa; the Schiff reagent and 2-hydroxy-3-naphthoic acid hydrazide stain the zona glomerulosa faintly or not at all (Fig. 7). Fixation of adrenals in formol-Zenker-osmic acid reveals osmiophilic lipid in glomerulosal cells as blackened droplets of variable size. The distribution of this lipid in the zona glomerulosa varies regionally to a considerable degree, with some clusters of glomerulosal cells containing much lipid (Fig. 11) and others little or none (Fig. 12). Alkaline phosphatase activity is absent from the zona glomerulosa after sections are incubated with

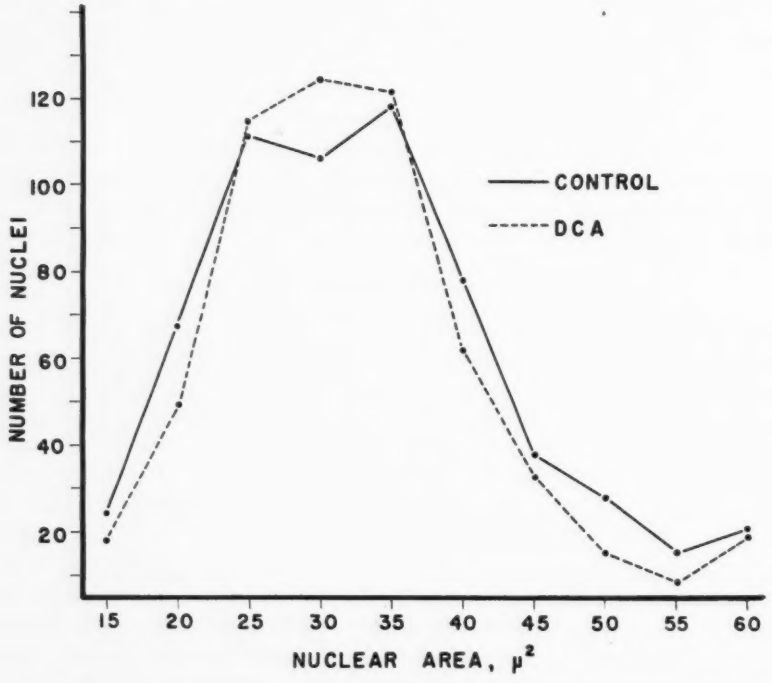

FIG. 1. Distribution curve of the nuclear areas of zona glomerulosa cells in adrenals of control hamsters and animals given 2 mg. of DCA daily for 10 days. This dosage of DCA does not produce a significant alteration of the distribution curve.

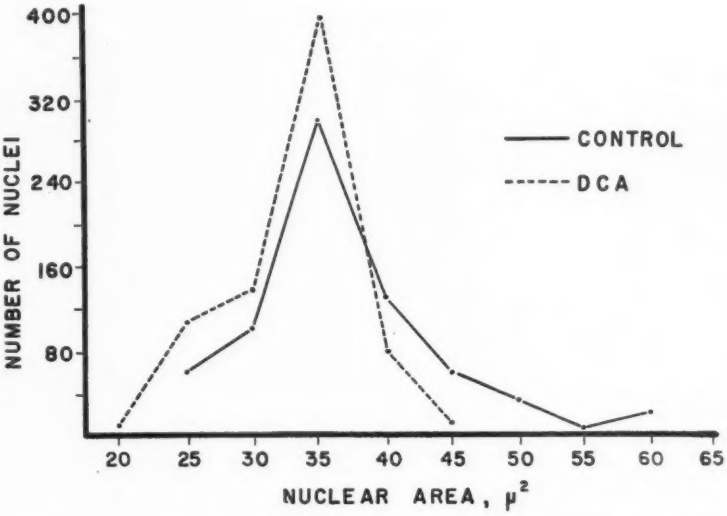

FIG. 2. Distribution curve of the nuclear areas of zona fasciculata cells. Two mg. of DCA produces a shift to the left in the curve, reflecting a decrease in average cross-sectional area.

sodium glycerophosphate for 1 or 3 hours at pH 9.2 (Fig. 5). Esterase activity, however, is more intense in this zone than in the remainder of the cortex (Fig. 9).

Administration of 2 mg. of DCA for 10 days does not affect significantly the architecture of the zona glomerulosa, the width of this zone remaining similar to that of control glands (Fig. 4). Mitotic figures are, however, observed less frequently than in controls. Because the cell boundaries of glomerulosal cells are difficult to distinguish clearly, the effect of DCA on cell size cannot be determined accurately. On the basis of the dimensions of the clusters of glomerulosal cells and the relative density of nuclei, there is no detectable change in cell size. The nuclei of glomerulosal cells are unaffected by DCA, the distribution curve of nuclear areas being similar to that of control glands (Fig. 1). Mitochondrial preparations do not demonstrate significant changes in glomerulosal cells after DCA treatment. Tests for sudanophilic lipid and cholesterol remain negative, while the Schiff reagent and 2-hydroxy-3-naphthoic acid hydrazide continue to stain the zona glomerulosa only faintly (Fig. 8). Osmiophilic lipid content varies regionally as it does in control glands and, when the entire zona glomerulosa in several sections of control and experimental adrenals are compared, there is no apparent effect of DCA on the concentration of this lipid. Alkaline phosphatase activity is negative, as it is in control glands; esterase activity is reduced only slightly after 2 mg. of DCA (Fig. 10).

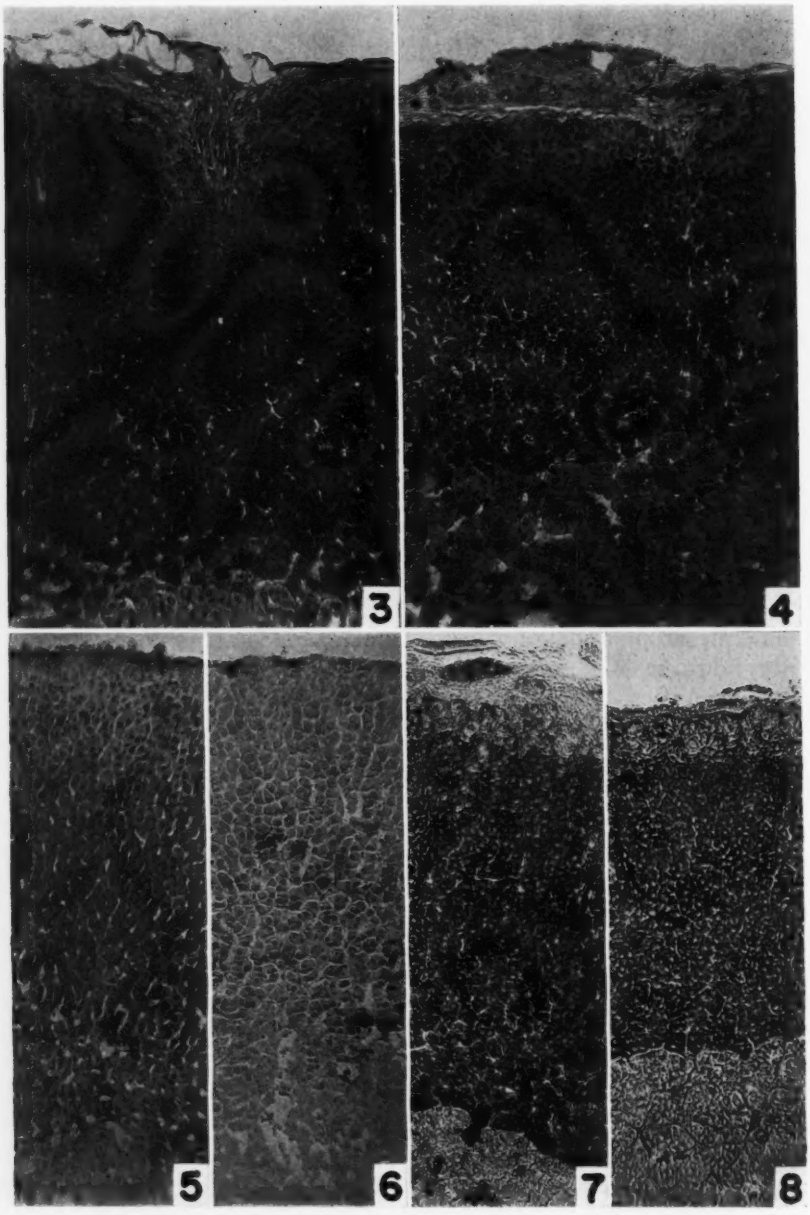

PLATE I

Zona fasciculata. In Bouin-fixed, Masson-stained adrenals of control hamsters, the zona fasciculata is composed of radially arranged columns of polyhedral cells, separated by narrow sinusoids (Fig. 3). A striking histological feature is the absence of cytoplasmic vacuolation, the fasciculata cells containing finely granular cytoplasm. A clear juxtanuclear, canalicular-like network, believed to be the negative immage of the Golgi apparatus, is seen occasionally. Fixation of adrenals in Regaud's fluid and staining with iron hematoxylin reveals that the cytoplasm of fasciculata cells is filled with small granular or rod-like mitochondria except in the juxtanuclear area of the negative Golgi apparatus (Fig. 15). In addition to mitochondria, there are present in each cell several bodies, identical to mitochondria in their staining, but which are larger, spheroidal or irregular in shape. These mitochondria-like bodies, or liposomes (Knigge, 1954a), are few in number in cells of the outermost fasciculata and increase in number in the inner portion of the fasciculata and zona reticularis. After formalin fixation, no sudanophilic lipid or cholesterol are demonstrated, but the Schiff reagent and 2-hydroxy-3-naphthoic acid hydrazide stain this zone intensely (Fig. 7). The reactive material is not removed by acetone extraction and is believed to be associated with the mitochondria (Knigge, 1954a). After fixation of adrenals in formol-Zenker-osmic acid, osmiophilic lipid droplets are not observed in normal fasciculata cells (Fig. 11). Moderate to intense alkaline phosphatase activity is demonstrated in the nuclei and cytoplasm of fasciculata cells, with the enzyme content of the outermost portion of the zona fasciculata being variable (Fig. 5). Esterase activity is present also, being more constant in its intensity and restricted to the cytoplasm of the fasciculata cells (Fig. 9).

Administration of 2 mg. of DCA produces an involution of the zona

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EXPLANATION OF PLATE I

FIG. 3. Adrenal cortex of a control hamster. The zona glomerulosa is narrow and its cells stain lighter than those in the zona fasciculata. Cells of the latter zone are not vacuolated. Bouin fixation, Masson stain. ×150.

FIG. 4. Cortex of a hamster treated with 2 mg. of DCA. The decrease in width of the cortex is due to reduction in size of the zona fasciculata cells, while the zona glomerulosa is unchanged. Technique as in Fig. 3. ×150.

FIG. 5. Alkaline phosphatase activity of a control cortex. The zona glomerulosa and outermost portion of the zona fasciculata exhibit weak enzyme activity. ×125.

FIG. 6. Alkaline phosphatase activity after 2 mg. of DCA. Enzyme activity in the zona fasciculata is reduced markedly. ×125.

FIG. 7. Control adrenal cortex, fixed in 10% formalin and stained with 2-hydroxy-3-naphthoic acid hydrazide. The zona fasciculata stains intensely while the zona glomerulosa is negative. ×125.

FIG. 8. After DCA, the staining with 2-hydroxy-3-naphthoic acid hydrazide is unchanged. ×125.

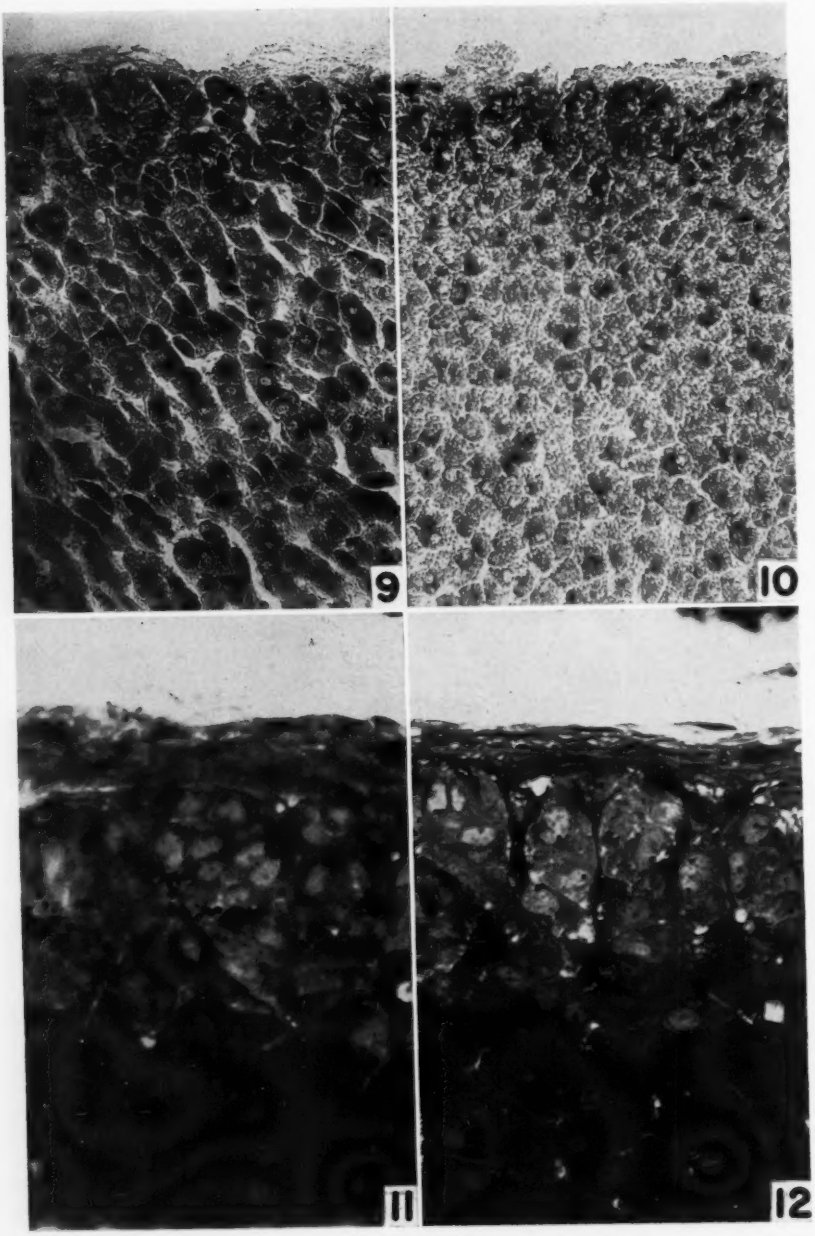

PLATE II

fasciculata, evidenced by a decrease in width of the cortex, and a decrease in the size of individual fasciculata cells (Figs. 4, 8, 14). The cytoplasm of these cells is somewhat more coarsely granular than normal, although the intensity of staining with eosin or the acid fushsin component of the Masson stain is not altered (Fig. 4). Cytoplasmic vacuolation, lipochrome pigment or cell degeneration are not observed. The nuclei of fasciculata cells become smaller and exhibit a shift to the left in the curve representing the distribution of their cross-sectional areas (Fig. 2). Mitochondrial preparations reveal that mitochondria in cells of the zona fasciculata are not altered significantly in adrenals of animals receiving 0.5 or 1 mg. of DCA. At a dosage of 2 mg., however, many cells contain mitochondria which are noticeably larger (Fig. 16), some of them being increased to such size that differentiation between mitochondria and liposomes is not possible. These intracellular bodies do not appear more densely packed than normal, and since the fasciculata cells decrease in size, the number of mitochondria in each cell would appear to be reduced. When sections of control and experimental adrenals are mounted on the same slide and stained with iron hematoxylin, the adrenals of DCA-treated animals stain lighter, suggesting that the mitochondria exhibit a reduced affinity for this stain. Tests for sudanophilic lipid or cholesterol are negative, the intensity of staining with the Schiff reagent and 2-hydroxy-3-naphthoic acid is unchanged (Fig. 8), and osmiophilic lipid droplets do not appear in the fasciculata cells. Alkaline phosphatase and esterase activity decrease, with 2 mg. of DCA producing the most marked reductions (Figs. 6, 10).

Kidney

The increase in kidney weight produced by DCA is due to an increase in the tubular parenchyma, as evidenced by fewer glomeruli seen per field in the treated animals as compared with the controls. After DCA, mitotic figures are observed more frequently in the proximal tubules, which exhibit

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EXPLANATION OF PLATE II

FIG. 9. Control adrenal cortex, fixed in 80% alcohol and stained for esterase activity. The zona glomerulosa exhibits greater enzyme activity than does the remainder of the cortex. ×200.

FIG. 10. After 2 mg. of DCA, esterase activity is reduced in the zona fasciculata while that in the zona glomerulosa is affected only slightly. ×200.

FIG. 11. Control adrenal cortex, fixed in formol-Zenker-osmic acid and stained with Altmann's acid fuchsin. Blackened osmiophilic lipid droplets are located in a characteristic position in the periphery of clusters of zona glomerulosa cells. No osmiophilic lipid is present in the zona fasciculata. ×650.

FIG. 12. Section of control cortex as in Fig. 11, showing an area in which the zona glomerulosa contains little or no osmiophilic lipid. ×650.

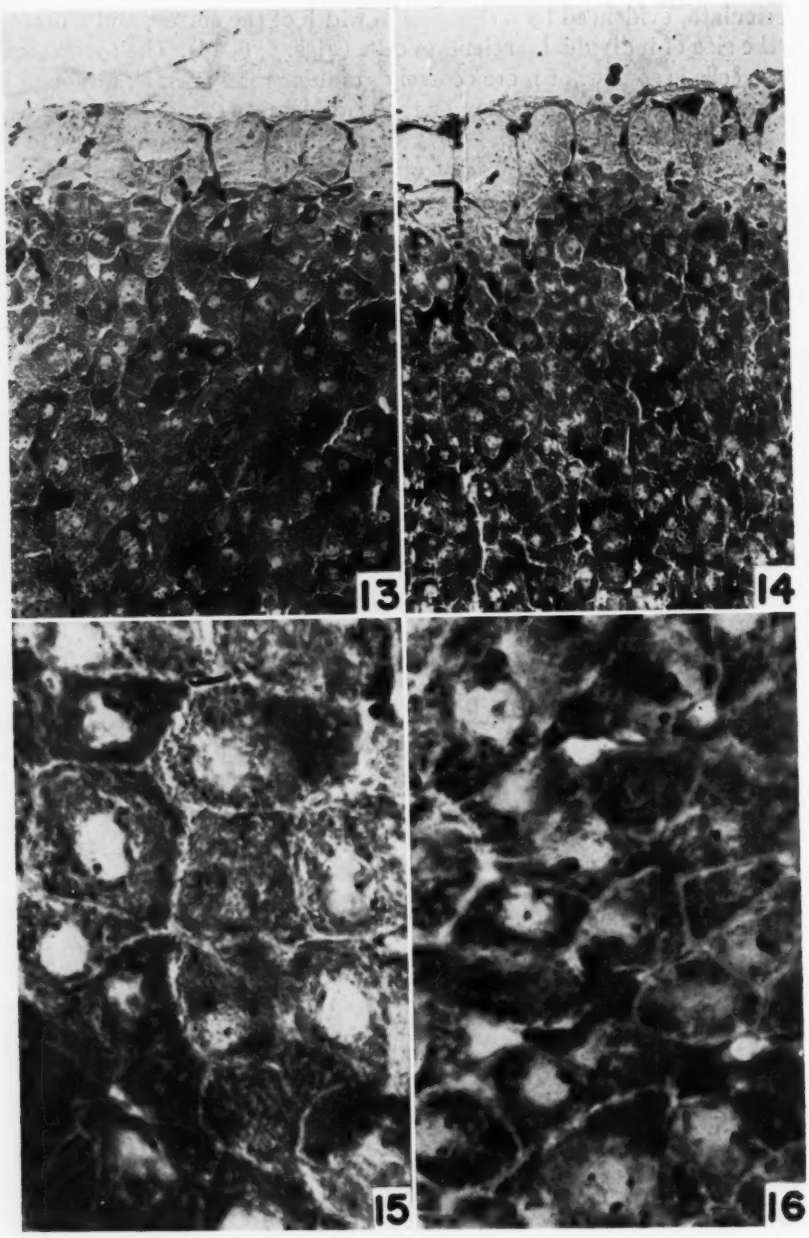

PLATE III

a slight increase in diameter (44.7 μ as compared to 38.5 μ for the controls). In animals treated with 2 mg. of DCA, the proximal tubules are considerably dilated, with the tubular epithelium decreased in height and the lumina containing marked amounts of granular debris, much of which represents desquamated epithelial cells. The kidney glomeruli of DCA-treated animals appear normal, although the range of sizes is larger than is observed in control animals.

Thyroid gland

The histology of the hamsters' thyroid is similar to that of other animals, consisting of rounded follicles of varying sizes, which are lined by a cuboidal epithelium. The average height of the epithelium in control animals is 10.6 μ.

Administration of 2 mg. of DCA does not alter the size of follicles or the staining properties of the colloid. The only detectable difference in histology of the follicular cells is an accumulation of small refractile, colloid-like droplets in the apical ends of the cells. The average height of the epithelium in animals given 2 mg. of DCA is 11.0 μ.

Hypophysis

The anatomical relationships, total volume and the relative size of the three principal parts of the hypophyses of male and female hamsters have been described (Knigge, 1954a). The relative per cent of acidophils, basophils and chromophobes in the control male hypophysis is 31.8, 14.2, and 54.0 respectively.

One-half and 1 mg. of DCA do not alter significantly the histology or relative per cent of pituitary cell types. Two mg. of DCA produces a decrease

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EXPLANATION OF PLATE III

FIG. 13. Control adrenal cortex, fixed in Regaud's fluid, sectioned at 3μ, and stained with iron hematoxylin. Mitochondria in cells of the zona fasciculata stain intensely while those in the zona glomerulosa are faintly colored. ×400.

FIG. 14. After 2 mg. of DCA, the width of the zona glomerulosa and the size of its constituent cell clusters are unchanged. Cells in the zona fasciculata are smaller and appear more coarsely granular. Technique as in Fig. 13. ×400.

FIG. 15. An area of the control zona fasciculata shown in Fig. 13. The cells are filled with mitochondria which are small, granular or rod-like. Nuclei are unstained. Technique as in Fig. 13. ×1200.

FIG. 16. Zona fasciculata of cortex shown in Fig. 14. After 2 mg. of DCA, the mitochondria in many cells appear as large spheroidal bodies, or liposomes. Technique as in Fig. 13. ×1200.

in the relative per cent of basophils (11.9%) and an increase in acidophils 35.0%), with many of the latter type appearing partially degranulated.

DISCUSSION

The zona glomerulosa in adrenals of hamsters is affected only slightly, if at all by DCA treatment. Cells in this zone do not exhibit a significant decrease in size and the distribution curve of their nuclear areas is unchanged; mitochondria are unaltered, esterase activity remains intense, and the osmiophilic lipid content appears normal. The bizonal theory of adrenal cortical secretion holds that mineralo-corticoids such as DCA are secreted by the cells of the zona glomerulosa, since in the rat this zone undergoes selective compensatory reduction of secretory activity when mineralo-corticoids are administered. The present study suggests that the hamsters' zona glomerulosa does not respond selectively to DCA, and indeed, experiments performed thus far on the hamster adrenal cortex do not conclusively demonstrate that glomerulosal cells are secretory in nature.

The progressive decrease in adrenal weight of hamsters given 0.5 to 2 mg. of DCA is due to a reduction of the zona fasciculata and zona reticularis, mediated probably through a decrease in secretion of corticotropin by the hypophysis. At the largest dose of DCA used, the adrenal weight is decreased by 33%. A state of reduced secretory activity is indicated because the cells of the zona fasciculata exhibit a decrease in size, a reduction in alkaline phosphatase and esterase activity, and a decrease in the size of their nuclei as reflected by a shift to the left in the distribution curve of their cross-sectional areas. Quantitative measurements of nuclear volume or cross-sectional area have been employed in detecting functional changes in adrenal cortical cells of several species subjected to various experimental conditions (Barkay, 1942; Overzier, 1950; Boguth *et al.*, 1951; Knigge, 1954b).

Mitochondria may reflect the secretory activity of the adrenal cortex (Greep and Deane, 1949; Miller, 1953), since they undergo hypertrophy with increasing secretory activity and decrease in number and size during states of hypoactivity. In the hamster, cells of the normal zona fasciculata are filled with small mitochondria. Thirty or 60 days after hypophysectomy, fasciculata cells are filled with spheroidal bodies, or liposomes, which exhibit all the staining properties of mitochondria, but which are many times larger (Knigge, 1954a). Evidence has been presented suggesting that such bodies represent altered or transformed mitochondria (Knigge, 1954b). In the present study, an apparent decrease in activity of fasciculata cells is accompanied by an increase in size of the mitochondria, although the number per cell is reduced. The increase in size of mitochondria after DCA administration, however, is not as marked as observed after

hypophysectomy. Other changes observed after treatment with DCA (adrenal weight, enzyme activity, etc.) indicate that the involution of the zona fasciculata is likewise not as marked as occurs after hypophysectomy. The fact that mitochondria do not transform into the typical, large liposomes of hypophysectomy further reflects an intermediate degree of atrophy produced by the dosage and time of administration of DCA used in the current experiment. Some index of the sensitivity of mitochondria as reflectors of cortical cell activity can be gained by noting that, although 0.5 and 1 mg. of DCA produce a significant decrease in adrenal weight, the mitochondria in such adrenals can not be differentiated with certainty from those in normal glands.

Changes in the hypophyses of hamsters given 2 mg. of DCA include a decrease in the relative number of basophils and an increase in acidophils. Administration of DCA or cortisone produces an increase in the corticotropin content of the rats' hypophysis (Cheng and Sayers, 1950; Tuchman-Duplessis, 1951a). It is not known whether an increase in corticotropin content occurs also in the hypophysis of hamsters given DCA, but the greater number of acidophils may reflect an increased storage. Changes in the secretion of thyrotropin, which is thought to be formed by basophils (Halmi, 1950; Purves and Griesbach, 1951), may be responsible for a decrease in this cell type in the hamster hypophysis after DCA, but there are no significant changes in the histology of the thyroid or the height of the follicular epithelium to suggest this.

SUMMARY

Young adult male hamsters were given 0.5, 1, or 2 mg. of DCA daily for 10 days, without affecting the weight of the body, heart or thymus. The adrenals exhibit a progressive involution, with 2 mg. of DCA producing a 33% weight loss. The zona glomerulosa does not appear to undergo a selective compensatory atrophy, in that the width of this zone, the size of its constituent cells and their nuclei, esterase activity, and osmiophilic lipid content are not altered by DCA. Cells of the zona fasciculata decrease in size and exhibit a reduction in alkaline phosphatase and esterase activity. Sudanophilic or osmiophilic lipid or cholesterol do not appear after DCA administration, while the staining with the Schiff reagent or 2-hydroxy-3-naphthoic acid hydrazide is unchanged. The nuclei of fasciculata cells decrease in size. The number of mitochondria per cell is decreased, but their size is increased.

Kidney weight is increased 55% by all three dosages. The height of the follicular epithelium of the thyroid is not altered significantly. Pituitary basophils are decreased, while acidophils are increased.

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THE EOSINOPENIC RESPONSE TO GRADED DOSES OF HYDROCORTISONE IN THE ADRENALECTOMIZED DOG WITH AND WITHOUT SURGICAL TRAUMA[1]

A. T. COWIE,[2] W. F. GANONG[3] AND D. M. HUME[4]

INTRODUCTION

IT HAS been suggested that the secretion of corticotrophin by the anterior pituitary is regulated by the blood level of adrenal steroids (Sayers and Sayers, 1948). This hypothesis, based on indirect experimental evidence, holds that in stress the body utilizes corticoids at a rate greater than normal, with resultant lowering of the blood level of adrenal corticoids. The pituitary, according to this concept, responds to this lowered level of adrenal steroids by increasing the secretion of ACTH.

In the present report, the eosinopenic response of adrenalectomized dogs to graded doses of hydrocortisone has been compared, with and without the addition of surgical trauma. If tissue utilization of corticoids by the traumatized animal were to lead to decreased blood corticoid levels, one might expect to see a lessened eosinopenia in the traumatized animal. This was not the case, however. A preliminary report of this work was made by Hume (1952).

MATERIALS AND METHODS

Ten to 15 kg. mongrel dogs of both sexes were used. They were fed a diet of horsemeat and Ken-L Biskit.[5] They were adrenalectomized in two stages a week to 10 days apart. At the second stage a 125 mg. desoxycorticosterone acetate pellet[6] was implanted subcutaneously, and the animals were maintained on added salt, one gram a day, for a period of one week. Subsequently they received no special treatment.

The dogs were tested by injecting a given dose of hydrocortisone into the femoral ar-

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[5] The Quaker Oats Company, Chicago, Ill.

[6] We are indebted to Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N. J. for the pellets of desoxycorticosterone (*Percorten*).

tery. Hydrocortisone acetate (Merck), 25 mg. per cc., was diluted with saline to achieve the desired concentration, and injected in a constant volume of one cc. of fluid. Blood for eosinophil counts was drawn into oxalate tubes before the start of the procedure and 2, 4, and 6 hours later. Absolute eosinophil counts were done according to the method described by Recant *et al.* (1950), with the exception that 0.1% eosin was used to make up the diluting fluid instead of 2%. Seven to 21 days later a "standard operation" was performed, and 10 minutes after the beginning of this trauma, the same dose of hydrocortisone was injected into the femoral artery above the site of the muscular trauma. A "standard operation" consists of making a 14 cm. incision through skin, subcutaneous tissue, and fascia of the hind leg, and dividing the underlying muscle in the direction of its fibers. The muscle edges are then retracted apart, and the muscle is freed up underneath. All bleeding points are tied off, and the wound is closed in layers with fine cotton sutures. The operation is done under pentothal-induced ether anesthesia and timed to take exactly 30 minutes. Eosinophil counts were taken as before, at 0, 2, 4, and 6 hours, starting with the beginning of the operation.

In some dogs more than one determination was done at each dose level, and the results were averaged. Not all dose levels were tested on all dogs, but whenever possible eosinophil falls with and without trauma for a given dose of hydrocortisone were paired. In addition, mean values for all determinations were obtained. The eosinophil falls at 2, 4, and 6 hours showed similar curves at the various dose levels, but since the eosinopenia was usually maximal at 4 hours, the 4 hour results only have been given in this report.

In four of the adrenalectomized dogs in this series "standard operations" were performed without the administration of hydrocortisone. "Standard operations" were also done in 9 other adrenalectomized dogs.

RESULTS

Table 1 summarizes the responses of these animals to various doses of hydrocortisone with and without surgical trauma. The results are computed on a log dose basis. It is apparent that surgical trauma does not decrease the eosinopenic effect of the injected hydrocortisone. Indeed, at lower dose levels, there is a tendency for the eosinophil fall with operation to be greater than it is without. There was considerable variation in the eosinophil counts as might be expected, and in a few instances, when the number of dogs used for the determination of a particular dose response was small, the standard error of the mean 4-hour eosinophil fall was large. However, the mean eosinophil counts all follow a consistent pattern. At the 0.3 mg. dose level, the mean 4-hour eosinopenic response to hydrocortisone plus trauma is significantly greater ($P < 0.001$) than the mean response to hydrocortisone alone. Regression lines were fitted by the method of least squares to both sets of data, using all the results at the various dose levels. These log dose response curves are shown in Figure 1, as are the mean 4-hour responses. The slopes of the two regression lines are significantly different ($P = 0.02$). Thus, while the lowest dose of hydrocortisone produces the greater eosinopenic response in adrenalectomized dogs when associated with trauma, increases in dosage produce *smaller* increments in the eosino-

TABLE 1

The mean 4-hour eosinophil fall in per cent at the various dosage levels of hydrocortisone with and without operative trauma in the adrenalectomized dog. In the last column the percentage difference between the eosinophil fall with trauma and that without trauma is given at the various dosage levels for those animals who had paired tests. There is a significant difference between the eosinophil fall with and without trauma at the lowest dosage level, whereas no effect of trauma is noted in the higher dosage levels. Operative trauma alone did not produce an eosinopenia.

Dose of hydrocortisone (mg.)	Operation	Mean 4-hour eosinophil fall (%)	Standard error of mean	Number of animals	% fall with operation minus % fall without (Paired tests only)
0.31	−	5.1	± 3.1	7	−22.5
	+	31.5	± 4.4	4	
0.63	−	22.8	± 6.1	10	− 7.9
	+	34.9	± 5.9	8	
1.25	−	34.7	± 8.9	7	− 4.3
	+	44.0	±20.4	3	
2.50	−	59.0	±11.2	7	+ 4.0
	+	47.8	± 7.7	6	
5.0	−	62.5	± 6.3	4	− 1.0
	+	63.7	± 4.7	3	

penic response when trauma accompanies the hydrocortisone than are obtained when no trauma is present. Therefore, when the higher dose levels are reached the responses converge, and no effect of trauma is evident.

When only the paired results are considered, the pattern of events is essentially the same, and the level of responses converge at the higher dose levels. (See Table 1.)

A "standard operation" *without* injected hydrocortisone was accompanied by a 10% eosinophil rise at 4 hours in the 4 animals in this series in which it was done. In a larger series of 9 other adrenalectomized dogs the 4-hour eosinophil change following "standard operation" was a 1% rise over the 0-hour value.

DISCUSSION

It has been fairly well established that in the adrenalectomized mouse, the eosinopenia produced by adrenal corticoids is roughly proportional to the dose until the eosinophil fall reaches a maximum (Speirs and Meyer 1951; Bibile 1953). Greater dosage cannot, of course, produce a fall greater than 100%, and thus the curve levels off. Our results confirm this impression, the log dose response being approximately a straight line over the dosage range of 0.3 to 5.0 mg.

The hypothesis on which this study was predicated was that if trauma caused increased body utilization of corticoids, less of the injected hydro-

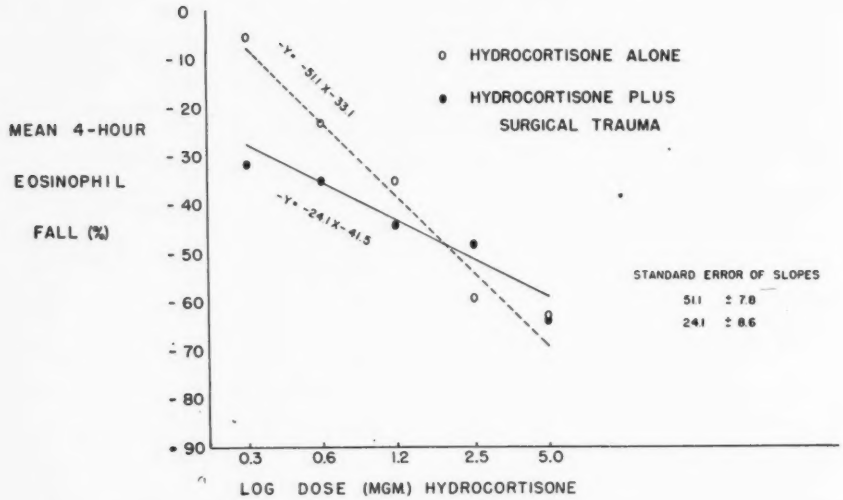

FIG. 1. The effect, in adrenalectomized dogs, of intra-arterial hydrocortisone on the blood eosinophil level with and without concurrent surgical trauma. The interrupted line is the log-dose response curve when hydrocortisone was given without trauma; the solid line is the log-dose response curve when it was given with trauma. It may be seen that at the lower dose levels there is a greater 4-hour eosinophil fall in the traumatized animals than in the non-traumatized animals. At the higher dose levels there is no significant difference between the two curves.

cortisone would be available to lower the eosinophil count, and accordingly, with operation, greater doses would be required to produce comparable eosinopenia. This, however, was not the case. Indeed, at the lower dose levels of hydrocortisone the eosinopenia was greater when trauma accompanied the hydrocortisone injection. This was particularly true of the lowest dose, which gave a minimal fall (5%) in non-traumatized adrenalectomized dogs, but a 31% fall when the animals were traumatized. At the higher dose levels trauma had no effect on the eosinopenic response to injected hydrocortisone. At first sight these results would appear to indicate that surgical trauma has a non-specific eosinophil-lysing effect independent of the adrenal hormones. This, however, is not the case, since the mean eosinophil change in 4 hours following surgical trauma without injected hydrocortisone in these animals was a 10% rise. Moreover, in a larger series of adrenalectomized dogs subjected to a standard surgical trauma similar to that used in this study the mean 4 hour eosinophil change was a 1% rise. Some other explanation for the increased eosinopenia with trauma must therefore be sought.

A simple explanation which would appear to fit the observed facts may

be tentatively put forward, although at present there are no experimental results to support it. It seems reasonable to assume that the minimal dose of hydrocortisone is rapidly inactivated or removed from the general circulation. If, however, the rate of inactivation or removal were retarded by some process such as the temporary depression of hepatic function incident to anesthesia and trauma, then the hydrocortisone would have a greater opportunity to effect the eosinophils. This would be particularly marked with minimal or near minimal doses, whereas with larger doses any decreased destruction of the corticoids would be relatively insignificant in comparison to the total quantity in circulation and so would not be reflected in the eosinopenic response. Hence the convergence of the results at the higher dose levels. In future work it is evident that the effect of anesthesia and trauma on corticoid conjugation and excretion must be further investigated.

In summary, our results indicate that there is no increased utilization of injected hydrocortisone in the traumatized adrenalectomized dog under the conditions of the experiment. Somewhat similar results have been obtained in humans by Thorn *et al.* (1953), who found that the amount of 17-hydroxycorticoids appearing in the urine of Addisonian patients after the injection of a given dose of cortisone was essentially the same with or without surgical trauma.

SUMMARY

1. The eosinopenic effect of graded doses of hydrocortisone injected into the femoral artery of the adrenalectomized dog has been determined, and has been compared with the eosinopenic effect of the same doses of hydrocortisone in the presence of surgical trauma to the injected limb.

2. With the lower dose levels of hydrocortisone a greater eosinopenic response was obtained in the presence of surgical trauma. It is suggested that this may be due to the decreased destruction or excretion of the injected corticoid as a consequence of hepatic depression incident to anesthesia and/or trauma.

3. At the higher dose levels surgical trauma did not significantly effect the eosinopenic response.

4. These findings fail to demonstrate an increased tissue utilization of corticoids by the injured animal.

5. These and other data throw doubt on the hypothesis that increased corticoid utilization is responsible for the pituitary release of ACTH in response to operative trauma.

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ENDOCRINE INFLUENCES ON THE DEVELOPMENT OF THE RUDIMENTARY GONAD OF FOWL[1]

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THE right gonad of the female domestic fowl ceases to grow at about the ninth day of incubation, when sex differentiation is about completed, and regresses subsequently until, at the time of hatching, only a small rudiment remains. In the mature domestic hen only the left gonad is fully developed and functioning as an ovary, but Brode (1928) was able to show conclusively that the right, rudimentary gonad is present as well.

Whenever the normal left ovary is removed surgically or is destroyed by disease, the right rudiment hypertrophies into an endocrinologically functional compensatory gonad which is always located in an area extending from the right adrenal caudad for a varying distance along the posterior vena cava. Rudiments may develop into testes, ovaries, or ovotestes. Domm (1927, 1939), working with Brown Leghorns reported that of 102 ovariectomies, 98% developed testis-like compensatory gonads, while the remaining 2% had ovotestes and ovaries.

The factors which suppress the rudiment in the presence of the left gonad have remained unknown. It is the purpose of this paper to report on the probable endocrine interrelation between the ovary and the rudimentary gonad of female domestic fowl.

MATERIALS AND METHODS

Female White Leghorn chicks were used in all experiments. Sinistral ovariectomies were performed from 5 to 27 days after hatching. The method employed in producing

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the poulards was essentially the one originated by Domm (1927). Birds were starved for 12 to 24 hours prior to the operation. Anesthesia was produced with intra-peritoneal nembutal, and, while the bird rested on its right side, an incision was made into the body cavity as close to the spine as could be done without damage to the kidneys from subsequent retraction. The ovary was brought into full view by pushing aside the intestine, its peritoneal covering was torn and the ovarian tissue removed as completely as possible by blunt dissection. Finally the whole area was electrocauterized in order to destroy all remnants of ovarian tissue. The surface of the left adrenal which is normally in contact with the ovary was also cauterized. While amount of cautery was held to a minimum, the possibility of damage to the rudimentary gonad from radiant heat cannot be excluded.

Poulards were assigned randomly to treatments and to compartments of a finishing battery. In studying the effects of hypophysectomy on the hypertrophied gonad, birds hatched two weeks apart were used. Poulards from each hatch were distributed randomly among treatments in order to achieve a uniform distribution of the two age groups. All hormones were injected subcutaneously, once daily. Steroids were dissolved in corn oil (Mazola) and pituitary hormones were made up in distilled water. Injection site was alternately the right and left pectoral region. In experiments involving the injection of steroid hormones only, all groups were injected with a like volume. The control group received corn oil only. In all other experiments the controls were not treated.

Initial and final body weights and comb sizes were obtained in all experiments. The product of comb length and comb height, recommended by Jones and Lamoreux (1943) as a sensitive index of the level of androgen secretion, was used to compare comb sizes.

At the termination of each experiment and within 12 to 24 hours from the time of the last injection, all birds were killed by luxation of cervical vertebrae. Macroscopically distinct rudiments and all structures found in the general location previously occupied by the ovary, and the oviducts were dissected out and fixed in Bouin's solution. After thorough fixation these tissues were superficially dried by blotting and weighed to the nearest milligram. All rudiments and all structures suspected of being gonadal regenerations were embedded in paraffin, sectioned at seven microns, mounted and stained with Milligan's (1946) trichrome stain. In characterizing the type of rudiment produced we have followed the convention of reserving the term ovotestes for the gonads containing follicles as well as medullary tubules with lumina.

Domm (1932) reported the perplexing incidence of oviducts distended with fluid. The watery secretion of these oviducts gives a positive Heller's ring test for albumin. These abnormal oviducts with thin and inelastic walls, occur regularly in a small number of poulards and they were excluded from our data. The weights of normal oviducts were used as an index of estrogen secretion.

RESULTS

Because the rudimentary gonad proliferates only in the absence of the ovary, it seemed logical to investigate if the growth of the compensatory gonad after ovariectomy could be prevented with exogenous sex hormones. Because the ovary secretes both androgen and estrogen, hormones representative of these groups were tested first.

While estrogen secretion cannot be determined by external inspection of live chicks, the response of the comb to androgen serves as a very convenient and sensitive criterion for estimating the time of onset of endocrine

function of the rudiment. Preliminary observations had indicated that reddening of the comb of White Leghorn poulards begins about 50 days after ovariectomy of the three week old birds. Based on this finding hormone therapy was initiated prior to that age.

Inhibition of growing rudiment by hormone implants. Birds ovariectomized at 8 to 18 days of age were used. Treatment was begun when the birds were 62 days old, before comb development had given evidence of gonadal function, and it was continued for 6 weeks. At the end of the experimental period all birds were sacrificed.

In this experiment we wanted to study the qualitative rather than the quantitative effect of ovarian hormones. For this reason hormone pellets of diethylstilbestrol (13 mg.) or of testosterone propionate (30 mg.) were implanted subcutaneously in the pectoral area. The pellets were dislodged once weekly to prevent encapsulation and they were replaced by new pellets after three weeks.

Experimental data, of which a preliminary account was given by Kornfeld (1953) and Kornfeld and Nalbandov (1953), are summarized in Table 1. Rudiments, too small to be macroscopically definite structures, were assumed to be completely inhibited and were arbitrarily assigned a weight of zero. Even so, the measurement of the weight of the smallest visible rudiments was frequently inaccurate, because unrelated connective tissue was removed with many of the structures which had poorly defined boundaries.

Differences between the mean rudiment weight of all three groups were highly significant ($P < 0.01$), indicating that both estrogen and androgen implants had depressed the compensatory gonad, but that estrogen was far more effective. Androgen did not increase oviduct weight significantly. Its effect upon comb growth was highly significant ($P < 0.01$) as expected. The effect of the diethylstilbestrol implants upon oviduct weight, comb growth, and gonad weight was highly significant ($P < 0.01$). The last two effects parallel these associated with the "physiological hypophysectomy" of males (Nalbandov and Baum, 1948).

Following estrogen treatment only 4 of 11 rudiments were visible to the naked eye and their mean weight was only 17 mg. The remaining 7 rudi-

TABLE 1. EFFECTS OF IMPLANTS OF ANDROGEN AND ESTROGEN ON GROWING RUDIMENTARY GONADS OF POULARDS

No. of observations	Treatment	Increase of comb area (%)		Rudiment weight (mg.)		Type of rudiment developed				Oviduct weight (mg.)	
		Mean	SD	Mean	SD	Testes	Ovaries	Ovotestes	None	Mean	SD
14 (13)*	None	197	88	92	54	10	1	3	0	208	192
11 (9)*	Estrogen	29	15	6	9	1	3	0	7	4488	5021
14 (13)*	Androgen	152.1	326	52	26	10	2	1	1	226	378

* Number of observations of oviduct weight (distentions omitted).

ments were assigned the weight of zero mg. It is perhaps interesting to note that microscopical examination showed that of the four rudiments only one was testicular. This ratio of 1:3 for estrogen treatment differs remarkably from the 10:4 for controls and 10:3 ratios for androgen treatment (Table 1). The possible significance of this finding will be discussed later.

Inhibition of growing rudiment by estrogen injection. While these results, recently confirmed by Taber and Salley (1954), conclusively demonstrated the inhibition of the growth of compensatory gonads of poulards with exogenous estrogen or androgen, the quantities of hormone used were admittedly unphysiological. In immature female chicks in which the ovary appears to be endocrinologically non-functional the rudiment does not develop beyond microscopic size. The oviducts in such chicks remain small and unstimulated until a few weeks before sexual maturity. If estrogens prevent the development of the rudiment in the normal, growing female, then they must be secreted in such small amounts that the oviduct is not stimulated. Because stimulation of the oviduct is the generally accepted criterion of estrogen production in birds, amounts, too small to affect that structure, are likely to remain undetected. It was the purpose of the next experiment to test the effect on the rudiment of estrogen levels which might occur *in vivo*.

Birds, ovariectomized at 9 to 15 days of age, were assigned to seven groups for the administration of graded dosages of estradiol cyclopentylpropionate, Upjohn (ECP). Dosages were adjusted every 10 days to the mean body weight of each group. The amounts of ECP injected per 100 gm. of body weight were: 0, 2, 4, 8, 16, 32 and 64 μg. respectively.

Treatment started about 15 days after ovariectomy when the birds were 31 days old and before the growing rudiment had begun to stimulate the comb visibly. The experiment was terminated when, after 72 daily injections, all birds receiving no estrogen had bright red combs, indicative of androgen secretion, and presumably, of gonadal function.

Again, rudiments, too small to be macroscopically definitive structures, were assumed to be completely inhibited and were arbitrarily assigned a weight of zero (Tables 2 and 3). Statistical analysis showed that maximal inhibition of the rudiment and the most pronounced changes in the type of rudiment produced, were already obtained on the lowest dose of estrogen used (Table 3). This indicates that we have failed to establish the minimum effective dose of estrogen in this experiment. There is a highly significant non-linear relation between oviduct weight and dose, which is linear for their logarithms (except for the points at doses 0 and 2 μg. which do not differ significantly). This indicates a threshold at about 3.4 μg. with 95% confidence limits at 1.6 and 7.0 μg.

Although the small number of observations precludes statistical signifi-

TABLE 2. EFFECT OF ESTROGEN ON THE DEVELOPMENT OF THE COMPENSATORY GONAD OF POULARDS

Daily ECP dose in μg./100 gm. of body weight	Total number of observations	Total number rudiments inhibited	Type of rudiment developed		
			Testes	Ovaries	Ovotestes
0	8	0	4	0	4
2	9	7	1	0	1
4	6	4	1	1	0
8	9	7	1	0	1
16	7	4	0	1	2
32	10	7	0	0	3
64	10	9	0	1	0
Total	59	38	Total:	21	

cance, it is interesting that no testicular compensatory gonad was observed on the higher dosages of estrogen (16 μg./100 gm. of body weight and above). This apparent estrogen effect parallels that observed in the first experiment and is similar to results obtained in embryos in which Willier *et al.* (1937) found that in general, irrespective of sex genotype, androgens tend to bring into expression the male tissue components while estrogen brings out the female ones.

Effect of estrogen on established rudiments. In the previous experiment we have shown that very small daily doses of estrogen inhibit the early growth of the rudimentary gonad of poulards, and it now became of interest to determine whether the established and functional compensatory gonad remains equally sensitive to estrogen.

Poulards which had been ovariectomized at 5 to 8 days of age, were allowed to become 147 days old before injections were started. The birds were assigned to four groups for the administration of graded dosages of ECP.

Because the oviduct weight of birds with ovaries and possibly with ovotestes (there was only one bird with an ovotestis among the controls and

TABLE 3. EFFECT OF GRADED DOSAGES OF ESTROGEN ON IMMATURE POULARDS

Number of observations	Daily ECP dose in μg./100 gm. of body weight	Increase in comb area (%)		Rudimentary gonad weight (mg.)		Oviduct weight (mg.)	
		Mean	SD	Mean	SD	Mean	SD
8 (8)*	0	232	57	79	47	128	59
9 (7)*	2	121	80	15	39	136	39
6 (5)*	4	92	20	13	26	187	102
9 (6)*	8	83	30	5	10	401	122
7 (7)*	16	60	33	12	15	619	295
10 (10)*	32	49	15	7	11	2942	1706
10 (9)*	64	45	34	10	31	4497	1469

* Number of observations of oviduct weight (distentions omitted).

TABLE 4. GRADED DOSAGE EFFECT OF ECP ON POULARDS WITH TESTICULAR COMPENSATORY GONADS

Number of observations	Daily dose† of ECP in μg./bird	Increase in comb area (%) during treatment		Gonad weight (mg.)		Oviduct weight (mg.)	
		Mean	SD	Mean	SD	Mean	SD
8 (7)*	0	79	68	394	300	2,413	3,850
7 (7)*	25	20	16	322	349	7,846	5,753
11 (9)*	50	52	88	255	175	8,507	4,747
6 (6)*	100	72	46	647	298	20,863	11,100

* Number of observations of oviduct weight (distentions omitted).
† The dose indicated was increased eight fold for the last 3 of 25 daily injections.

its oviduct weighed 7,545 mg.) suggested a different level of endogenous estrogen from the rest of the group, and because their small number was not evenly distributed among all treated groups, data from birds with non-testicular gonads were not included in the statistical analysis. Because these birds were no longer growing rapidly (their mean body weight increased about 10% during the period of treatment), it was not considered necessary to adjust dosages to body weight. For ease of comparison with the data of the earlier experiment, it should be stated that the group administered the highest dose never received less than 6 μg. ECP/100 gm. of body weight/day.

An analysis of covariance indicated no significant difference between mean gonad weights even after adjustment for initial comb size (Table 4). This means that in this experiment ECP at three times the daily level which inhibits growth of the rudiment, had no effect upon the established compensatory gonad.

Some degree of spermatogenesis was encountered in 25% of the testicular rudiments and it was apparently not affected by treatment (Table 5). Spermatogenesis was frequently restricted to some areas of the gonad, while the rest of the structure consisted of sterile tubules and of cords of

TABLE 5. TYPES OF COMPENSATORY GONAD OBSERVED

Daily dose of ECP in μg./bird*	Number of				
	Testes			Ovaries	Ovotestes
	No spermatogenesis	Incomplete spermatogenesis	Complete spermatogenesis		
0	6	1	1	2	1
25	5	1	1	2	1
50	10	1	0	1	0
100	3	2	1	0	2

* The dose indicated was increased eight fold for the last 3 of 25 injections.

epithelial cells which, according to Gray (1930), are their precursors. Among the ovaries, four had ovulatory follicles and empty follicles which gave proof of past ovulations.

Estrogen in chicken blood. In the young female chick the oviduct remains infantile from hatching until the bird is 70 to 80 days of age suggesting absence of endogenous estrogen. Because the data obtained (Table 2) indicate that exogenous estrogen does inhibit the growth of the rudimentary gonad *in vivo*, and because it is known that in the normal immature female the rudiment does not hypertrophy, the question arose whether endogenous estrogen below the threshold level of oviducal response may be produced by immature female chicks. An assay of the estrogen content of the blood of young female chicks was made.

Female chicks 16 to 20 days old were exsanguinated, their blood pooled and extracted with ethylene dichloride (Hays, 1949). For the assay, 27-day old ovariectomized rats were given nine equal subcutaneous injections in four and one half days. Twelve hours after the last injection the rats were killed, the uteri removed and trimmed; uterine fluid expressed by light blotting and the uteri weighed to the nearest 0.1 mg. The results of the assay permit only a qualitative interpretation but they do indicate that appreciable quantities of estrogen are present in the blood of immature female chicks less than 20 days old (Table 6). A small quantity of chick ovaries which were extracted by the same method contained no estrogen. The extraction procedure may have been unsuited for small quantities of tissue and no conclusions are being drawn from the failure to find estrogen in these ovaries.

The role of the pituitary gland and of gonadotrophic hormones. To determine the role of the pituitary gland and of some of its hormones in the growth of the rudiment, two experiments were performed.

In both experiments potent gonadotrophic hormones containing FSH and LH were used. The same preparations had produced very pronounced effects when they had been used in previous experiments in normal male and female chickens. The hormones used were: An Armour gonadotrophic preparation which was injected at the rate of 2 and 4 Armour units daily;

TABLE 6. BIOASSAY OF THE ESTROGENIC ACTIVITY OF AN EXTRACT OF NORMAL CHICK BLOOD

Treatment (total dose)	Number of rats	Uterine weight	
		Mean	SD
Controls	5	53.1	8.7
Extract of 37.8 gm. of pooled blood	5	183.2	27.0
0.18 μg. estradiol benzoate	5	141.8	11.2
0.36 μg. estradiol benzoate	5	167.7	15.4

TABLE 7. EFFECTS OF HYPOPHYSECTOMY OF POULARDS WITH HYPERTROPHIED COMPENSATORY GONADS AND ATTEMPTS AT REPLACEMENT THERAPY

Number of observa-	Treatment (all ovariectomized)	Change in comb area (%) during treatment		Gonad weight (mg.)		Oviduct weight (mg.)	
		Mean	SD	Mean	SD	Mean	SD
14 (10)*	None	+17.49	10.02	479	282	740	602
11 (8)*	Hypophysectomized	−44.23	6.12	61	56	471	488
3 (3)*	Hypophysect. + lyophilized plasma	−36.29	11.23	40	28	127	38
2 (2)*	Hypophysect. + Gonadotroph (1) then prolactin	−31.68	14.63	68	46	112	30
3 (3)*	Hypophysect. + Gonadotroph (1) then Gonadotroph (2)	−30.47	11.13	119	73	111	36
4 (3)*	Hypophysect. + Gonadotroph (1)	−29.06	5.54	88	23	123	34

* Number of observations of oviduct weight (distentions omitted).
Gonadotroph (1) Armour preparation.
Gonadotroph (2) Wisconsin preparation.

a crude anterior pituitary gland preparation (sheep) which contained both FSH and LH and which was given at the rate of one gm. equivalent per day; pregnant mare's serum, which was injected at the rate of three Cartland-Nelson units per day; and a lyophilized chicken blood plasma which was thought to contain an avian gonadotrophic hormone (Bailey and Phillips, 1952) but which, according to a rat assay, had very little gonadotrophic potency. It was given at the rate of 0.5 mg. per day. In addition to these hormones we were interested in seeing the effect of a prolactin preparation and injected a Squibb product containing 20 to 25 crop gland units per mg. at the rate of 10 mg. per day, and, in one experiment at the rate of 40 mg. per day.

In the first experiment, poulards with established rudiments were hypophysectomized and an attempt was made to prevent postoperative regression of the rudiment and hence the comb by the injection of gonadotrophic hormones. This experiment was done on birds in which the ovary had been removed at 15 to 27 days and which were hypophysectomized when they were between 170 and 184 days old. For this experiment poulards were selected on the basis of characteristics usually associated with maleness: turgid, red combs and male plumage. It was felt that the effects of hypophysectomy, never before observed in poulards, would be easier to interpret in a group homogenous in external morphology and, presumably, in type of gonad. At autopsy it was found that all the birds thus selected had testes and that we had eliminated all the poulards having cortical gonadal regenerations, right ovaries, or ovotestes because these showed predominantly female external characteristics. None of the gonadotrophic

hormones used was able to retard the rate of regression of the hypertrophied rudiment or of the comb after hypophysectomy of the poulards (Table 7). This experiment did, however, establish the fact that the rudiment regresses in size and in function in absence of the pituitary gland just as do the testes and ovaries of normal chickens after hypophysectomy.

In a second experiment it was attempted to speed up the development of the rudimentary gonad in poulards which had been ovariectomized when 9 to 16 days old, by injecting gonadotrophic hormones beginning at the age of 34 days and continued for 31 days. Gonadotrophic hormones were not able to speed up the rate of rudiment growth in young poulards as judged by the final weight of the rudiment, or the rate at which it secretes androgen as judged by the rate of comb growth (Table 8). Nor do the hormones injected alter the type of gonad produced (Table 8). Of interest is the finding that prolactin depressed the development of the rudiment causing a final gonad weight significantly ($P < 0.05$) below that of untreated poulards or that of poulards given gonadotrophic hormones (Table 8). Prolactin also depresses the size and rate of function of the gonads of normal male and female chickens.

DISCUSSION

The results presented indicate that small amounts of exogenous estrogen can suppress the development of the rudimentary gonad of ovariectomized fowl. The demonstration of substantial quantities of estrogen in the blood of very young female chicks, makes it probable that a hormone of this type alone or by a synergism yet unknown, is responsible for the failure of the development of the rudimentary gonad in the presence of the functional left ovary. The fact that hypophysectomy of birds with established compensatory gonads leads to a significant decrease in the size of the rudiment indicates that a pituitary factor may also be involved. We were not able to prevent this regression by the administration of massive doses of mammalian gonadotrophic hormones. Nalbandov *et al.* (1951) have shown that in hypophysectomized male chickens a third gonadotrophic hormone is re-

TABLE 8. EFFECTS OF HORMONE THERAPY ON IMMATURE POULARDS

Number of observations	Treatment	Increase in comb area (%) during treatment		Final comb color		Gonad weight (mg.)		Structure of rudimentary gonad		Oviduct weight (mg.)	
		Mean	SD	Red	Pale	Mean	SD	Testes	Ovo-testes	Mean	SD
10 (5)*	None	228	71	4	6	28	11	7	3	75	20
8 (6)*	PMS	207	59	7	1	26	7	4	4	60	12
7 (7)*	Progesterone	269	88	5	2	28	10	5	2	99	47
7 (5)*	Prolactin	141	110	0	7	17	11	5	2	74	38
5 (3)*	LH	182	77	1	4	29	5	4	1	74	81

* Number of observations of oviduct weight (distentions omitted).

quired for the transformation of non-secretory Leydig cells into secretory one and that mammalian gonadotrophins alone are not fully effective in maintaining the ovaries and the comb of hypophysectomized laying hens (Biswal and Nalbandov, 1952). It remains to be seen whether a mechanism, involving a third gonadotrophic hormone operates in the interrelationship between the pituitary and the rudimentary gonad.

While it has been shown that estrogens effectively inhibit the pituitary gland in chickens (Nalbandov and Baum, 1948), it is not known whether the controlling quantities are as small as they have been found to be in the rat (Byrnes and Mayer, 1951). If the avian hypophysis is sensitive to minute quantities of estrogen, it appears quite possible that the inhibitory effect of estrogen on the rudimentary gonad is not direct, but operates via the pituitary by reducing the flow of the whole gonadotrophic complex or of a specific component of it. That this interpretation may be correct is further suggested by the reduction in size and function of the rudiment following hypophysectomy.

Further support for the assumption that hypophyseal gonadotrophins have some measure of control over the proliferation of the rudiment, was obtained from the finding that prolactin significantly depresses the weight of the rudiment. This is in keeping with the finding of Nalbandov (1945), that in cocks prolactin reduces both testes and comb size in proportion to the amount of prolactin injected. The FSH portion of this effect on cocks may be nullified by the simultaneous injection of prolactin and FSH. This indicates that prolactin prevents the secretion of pituitary FSH but that it does not prevent the action of FSH on its endorgan. The inhibiting effect of prolactin on the rudimentary gonad may be due to a similar mechanism (although we were not able to maintain the rudiment in hypophysectomized poulards with mammalian gonadotrophic hormones).

Because of these considerations the following interpretation appears reasonable at the present time. Like all gonadal tissues, the rudimentary gonad of the fowl is normally controlled by hypophyseal hormones. In the presence of the normal ovary, the rudiment is, by some as yet unknown mechanism, effectively inhibited by ovarian secretions. The net effect of this interrelation is that the rudimentary gonad is incapable of developing in the intact female, but does hypertrophy following ovariectomy when it is released from estrogen inhibition and exposed to the stimulating effect of pituitary gonadotrophins.

SUMMARY

The growth of the rudimentary right gonad of ovariectomized, domestic fowl can be inhibited with exogenous estrogen and, to a lesser extent, with androgen. It is possible to prevent the hypertrophy of the rudiment even

with a quantity of estrogen that has no significant effect upon oviduct weight and which apparently lies below the estrogen threshold for oviduct stimulation.

Estrogenic substance has been demonstrated in the blood of young chicks. The adequacy of a "physiological" dose of estrogen coupled with the demonstration of its presence at an age of sexual "inactivity," lends strong support to the contention that estrogen is involved in the *in vivo* mechanism of rudiment inhibition.

Following ovariectomy the sensitivity of the compensatory right gonad decreases with time, i.e., estrogen dosages increased in proportion to body weight lose their effectiveness. This observation helps to explain that compensatory gonads can, and ultimately do secrete considerable amounts of estrogen.

Prolactin depresses rudiment hypertrophy in ovariectomized birds. The mode of action of prolactin, known to be antagonistic to that of FSH, might be analogous in effect at least, to the mechanism of rudiment inhibition by estrogen.

Hypophysectomy significantly depresses the weight of hypertrophied testicular rudiments as well as their androgen secretion.

All attempts to accelerate the growth of compensatory gonads, or to maintain them in hypophysectomized poulards with mammalian gonadotrophic hormones have failed.

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A COMPARISON OF THE BIOLOGICAL ACTION OF ALLOPREGNANE-21-OL-3,20-DIONE ACETATE AND PROGESTERONE[1]

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THE inhibitory effect of progesterone on the estrogen-induced tissue growth response of the chick oviduct was reported by Hertz *et al.* (1947). Among other steroid hormones tested for inhibitory activity in the chick oviduct test, allopregnane-21-ol-3,20-dione acetate (cpd. I) proved to be a potent antagonist (Hertz, 1951). In a test for corticoid activity, Wettstein and Hunzicker (1940) reported the compound to be inactive for weight maintenance and survival in adrenalectomized rats at a daily dose of 2 mg.

Since progesterone and cpd. I inhibit the estrogen-stimulated chick oviduct, further investigations were made to determine whether these compounds possessed any other common biological properties.

In the present report, we present details of a comparison between progesterone and cpd. I based on certain biological actions exhibited by progesterone.

MATERIALS AND METHODS

(1) *Oviduct inhibition test*

Two week old New Hampshire Red chicks were housed in electrically heated brooders under controlled lighting (12 hours darkness, 12 hours light). Commercial starting mash was given *ad libitum*. 0.25 mg. stilbestrol in 0.2 ml. corn oil solution was administered daily subcutaneously for eight days. Progesterone and allopregnane-21-ol-3,20 dione acetate (cpd. I) were given as suspensions in 0.2 ml. corn oil daily subcutaneously concurrently with the stilbestrol treatment. Twenty-four hours after the last injection, the chicks were autopsied. Body and oviduct weights were recorded.

(2) *Corner-Allen test*

Previously isolated, sexually mature female New Zealand white rabbits maintained on a commercial pellet diet were used. The animals were bilaterally ovariectomized, a biopsy of the uterine cornu was taken and injections of the test compound were begun on completion of operative procedures. The test compounds in corn oil suspension were injected daily subcutaneously for 5 days. Twenty-four hours after the last injection the animals were killed. The uteri were fixed in 4% neutral formalin, sectioned at 8 μ, and stained with Mayers' hematoxylin and eosin Y.

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[1] We wish to thank Chemical Specialties Co. and Syntex, S.A. for the allopregnane compound used in these experiments.

(3) *Rat uterine deciduoma test*

Female Holtzman strain rats maintained on stock rat pellets were bilaterally ovariectomized at 28 days of age. Six weeks later treatment was begun. Daily injections of the test compound were given in 0.2 ml. corn oil suspension for 9 days. On the fifth day of treatment, the uteri were traumatized by means of a needle passed through the antimesometrial axis of each uterine cornu twice, with a #6.0 silk suture identifying the area of trauma.

The animals were autopsied 20 to 24 hours after the last injection. Body and uterine weights were recorded. Sections of the traumatized uterine areas were fixed in 4% neutral formalin and prepared for histological study by the methods described in Section 2.

(4) *Suppression of pituitary gonadotropin*

Thirty day old female Holtzman strain rats were put in parabiosis according to the method of Bunster and Meyer (1933) as modified by Hertz and Meyer (1937).

The left partner was ovariectomized at the time of operation. In the treated pairs, the left partner was injected daily subcutaneously with cpd. I or progesterone in 0.2 ml. corn oil suspension for 10 days. On the eleventh day the animals were killed with chloroform and autopsied. Ovaries and uteri were weighed to the nearest milligram on a torsion balance.

(5) *Effect on estrogen-withdrawal bleeding in the monkey*

Castrate female rhesus monkeys, weighing 3–4 kg., were treated with 10 micrograms estradiol 17-beta in 0.25 ml. corn oil solution daily subcutaneously for 20 days. Treatment with the test compound was begun on the twenty-first day and continued until menstrual bleeding occurred after estrogen priming. Daily injections of the test compound were made subcutaneously in a 1.0 ml. aqueous suspension. Menstrual bleeding was determined by daily vaginal smears.

RESULTS AND DISCUSSION

(1) *Inhibition of estrogen stimulation in the chick oviduct*

The inhibitory action of allopregnane-21-ol-3,20-dione acetate (cpd. I) on the genital tract of the estrogen-treated chick is compared with that of progesterone in Figure 1. Increasing doses of each compound result in progressive decreases in oviduct weight up to a daily dose of 1 mg. With daily levels of 1.0 mg. and above, it is apparent that either compound is maximally effective in its ability to suppress growth of the oviduct. However, at daily dose levels of 0.25 mg., cpd. I is decidedly more potent than progesterone. Comparing oviduct weights at 0.25 mg. and 0.5 mg. of cpd. I with weights attained with 1.0 mg. of progesterone, it would appear that cpd. I possesses 2 to 4 times the inhibitory activity of progesterone with respect to the estrogen-induced growth of the chick genital tract.

(2) *Progestational proliferation of the rabbit endometrium (Corner-Allen method)*

Cpd. I and progesterone were compared using total dose levels of 1.0 mg. in one series and 125 mg. in the second. Four rabbits were used for each

TABLE 1. ABSENCE OF DECIDUAL RESPONSE IN RATS TREATED WITH ALLOPREGNANE-21-OL-3,20-DIONE ACETATE

No. of animals	Steroid	Daily dose (mg.)*	% deciduoma response
10	Progesterone	0.5	90
9	Progesterone	1.0	100
10	Cpd. I	0.5	0
10	Cpd. I	1.0	0
10	None	0	0

* Given in 0.2 ml. corn oil suspension for 9 days.

dose. Histological examination showed no endometrial proliferation in the animals receiving cpd. I. This was in marked contrast to the full progestational proliferation produced in both groups receiving progesterone.

(3) *Deciduoma response*

Results of testing cpd. I for its ability to induce the formation of uterine deciduomata are seen in Table 1. It is evident that at the doses used, cpd. I does not produce the typical decidual reaction so characteristic of progesterone.

(4) *Effect on endogenous gonadotropin*

Table 2 contains a summary of experiments using castrate female-intact female parabiotic rats to assess the ability of cpd. I to inhibit gonadotropin production by the castrate partner. No pituitary gonadotropin inhibition occurred. Using rats of the same strain and age, a daily dose of 5 mg. of progesterone given for ten days results in ovarian weights of 23 ± 5 mg.[2] This inhibition of gonadotropic hormone secretion by progesterone has been reported by Biddulph *et al.* (1940).

TABLE 2. LACK OF EFFECT OF ALLOPREGNANE-21-OL-3,20-DIONE ACETATE ON OVARIAN HYPERTROPHY IN CASTRATE FEMALE-INTACT FEMALE PARABIOTIC RATS

Series	No. of pairs	Dose (mg.)*	Ovarian wt. (mg.)†‡
I	8	2	147 ± 55
	12	0	174 ± 58
II	9	10	157 ± 35
	4	0	136 ± 19

* Daily dose given in 0.2 cc. aqueous suspension for 10 days.
† Mean and standard deviation.
‡ See text above for progesterone effect.

(5) *Effect on estrogen withdrawal bleeding in the monkey*

A group of 4 female monkeys each received 1 mg. cpd. I daily following estrogen priming. Withdrawal bleeding occurred in three monkeys after four days of treatment and in the fourth animal on day seven. A 50 mg.

[2] Represents 10 parabiotic pairs.

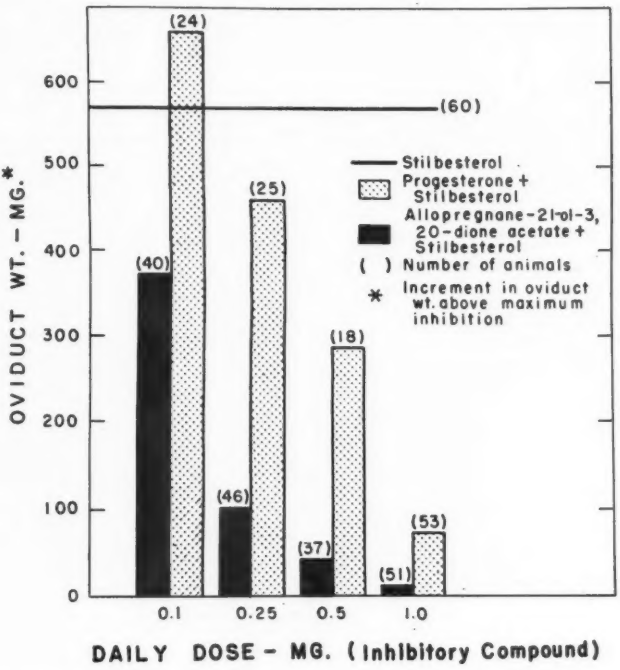

FIG. 1. Inhibition of estrogen stimulated chick oviduct growth. A comparison of allopregnane-21-ol-3,20-dione acetate with progesterone.

daily dose of cpd. I was given to each of a second group of 4 monkeys following estrogen treatment. Menstrual bleeding occurred in two of these animals on the second day, one on the third, and one on the fourteenth day of treatment. Of 6 monkeys receiving no treatment after estrogen priming, withdrawal bleeding occurred between the third and tenth days. We have confirmed previous reports by Hisaw and Greep (1938) that as little as 1.0 mg. progesterone daily will delay withdrawal bleeding in the rhesus monkey from 9–44 days. Our results indicate a lack of progestational activity for cpd. I at levels equal to or 50 times greater than the effective dose of progesterone.

The results show that in these experiments in which progesterone is known to have biological activity, the inhibition of the chick oviduct response to estrogen is the only positive demonstration of activity by cpd. I (Fig. 2). Thus we have a unique action for this steroid which is similar to that of progesterone but dissociated from the other known physiologic effects of the latter.

This inhibitory action of progesterone and compound I has yet to be ex-

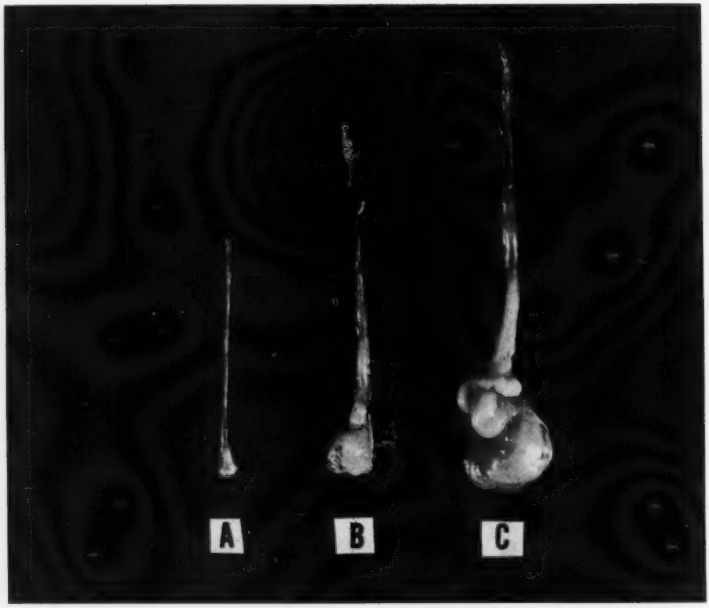

FIG. 2. A. Oviduct from untreated chick, wt. 23 mg.; B., oviduct from chick receiving 1 mg. allopregnane-21-ol-3,20-dione acetate plus 0.25 mg. stilbestrol daily for eight days, wt. 361 mg.; C., oviduct from chick receiving 0.25 mg. stilbestrol daily for eight days, wt. 1125 mg.

plained. It has previously been shown that only part of the estrogen-induced oviduct growth can be prevented by progesterone (Hertz *et al.*, 1947). Cpd. I, even though more potent than progesterone, is unable to reduce this basal oviduct growth below that reached by maximally effective doses of progesterone. Large doses of estrogen do not reverse this effect of progesterone on the oviduct. This indicates an irreversible reaction.

Lipschütz *et al.* (1939) demonstrated the antifibromatogenic action of progesterone on uterine and abdominal fibroids produced by prolonged estrogen stimulation in the guinea pig. Cpd. I has not been tested[3] for antifibromatogenic action but many other steroids have been screened (Lipschütz, 1950). Allopregnane-3,20-dione is negative at doses ten times those of the effective doses of progesterone (Lipschütz, 1950). Desoxycorticosterone is among the most active of these tumor inhibiting compounds (ibid.).

Desoxycorticosterone also inhibits growth of the estrogen stimulated

[3] Personal communication with Dr. A. Lipschütz.

chick oviduct (Hertz and Tullner, unpublished data). In this connection, it is of interest that Schneider (1952), using *in vitro* methods, has recovered cpd. I among the metabolites produced when desoxycorticosterone was incubated with surviving rat liver slices.

Although the effect of these inhibitory compounds on the substrate is unknown, it is clear that the inhibitory effect may be dissociated from other biological effects.

SUMMARY

A comparison of allopregnane-21-ol-3,20-dione acetate with progesterone on the basis of the well-known actions of the latter compound in (a) inhibiting the estrogen-induced growth of the chick oviduct, (b) progestational activity in the rabbit uterus, (c) deciduoma formation in the rat uterus, (d) suppression of pituitary gonadotropin in parabiotic rats and (e) prevention of withdrawal bleeding in estrogen-primed castrate female monkeys, has demonstrated that inhibition of chick oviduct growth is the only action common to the two steroids. Moreover, allopregnane-21-ol-3,20-dione acetate was found to be 2 to 4 times as potent as progesterone in the oviduct assay. Thus, inhibition of the oviduct in the estrogen-treated chick may be clearly dissociated from other biological actions of progesterone.

Acknowledgment

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A QUANTITATIVE METHOD FOR THE SEPARATION OF THE IODINATED AMINO ACIDS OF THYROID TISSUE[1]

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THE ability of a kieselguhr column to resolve mixtures of thyroxine, triiodothyronine and iodide (Grönkvist and Hellberg, 1951; and Gross and Pitt-Rivers, 1952) suggested that this column might be superior to starch (Dobyns and Barry, 1953) and cellulose (Rosenberg, 1951) columns for separating iodinated amino acids. In this paper we shall describe and evaluate an extension of the Gross and Pitt-Rivers kieselguhr technic which allows accurate separation and recovery of all the known iodinated compounds of the thyroid gland. In addition we have applied this technic to an evaluation of certain methods of hydrolysis of thyroid tissue.

METHODS

For the separation of thyroxine, triiodothyronine and iodide, the kieselguhr column procedure of Gross and Pitt-Rivers (1953), with a few exceptions, has been followed. We have not found that it is necessary to heat the kieselguhr[3] prior to use. Glass columns with an internal diameter of 13 mm. were used. The lower part of the kieselguhr column was prepared by mixing 6 gm. of kieselguhr with 4.8 ml. of 0.5 N sodium hydroxide saturated with 25 per cent chloroform in n-butanol (v/v). Twenty-five per cent chloroform in n-butanol (v/v) saturated with 0.5 N sodium hydroxide was then added to the mixture until a thin slurry was obtained. This slurry was degassed on a water pump and packed in the glass column with a perforated disk to a height of approximately 15 cm. Approximately 0.3 gm. of Gooch crucible asbestos was next packed on top of this first layer of kieselguhr. The mixtures to be separated, contained in 0.5 ml. of 0.5 N sodium hydroxide, 1.0 ml. of n-butanol saturated with 0.5 N sodium hydroxide or 0.8 ml. of a butanol-ethanol-sodium hydroxide mixture (see below), were added to the asbestos and washed into the column with two small portions of the butanol-chloroform mixture. Another layer of kieselguhr (5 gm. of kieselguhr mixed with 4 ml. of base saturated with 20% chloroform in n-butanol) was then added on top of the asbestos in a similar manner.

The elution of thyroxine, triiodothyronine and iodide respectively was accomplished

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[1] Abridgment of part of a thesis submitted by Dr. Braasch to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Surgery.

[2] Fellow in Surgery, Mayo Foundation. The Mayo Foundation is a part of the Graduate School of the University of Minnesota.

[3] Super-cel hyflo, Johns Manville.

by passing a 20% mixture of chloroform in n-butanol (v/v) saturated with 0.5 N sodium hydroxide through the column at such a rate that the flow was approximately 1 ml. every 5 minutes. This constant flow was maintained by using a rubber-stoppered separatory funnel as a reservoir for the solvent and placing the open delivery tip of the funnel at a level above the column appropriate to give this flow rate. After elution of these three substances, the solvent was changed to a 9:1 n-butanol to n-propanol mixture saturated with one part 0.5 N sodium hydroxide. Diiodotyrosine was then eluted followed by and well separated from monoiodotyrosine. Any residual material on the column (column residual) was eluted by water or propanol.

The effluent was collected in 3-ml. fractions with a fraction collector. Identification of compounds in the effluent samples was made both by ninhydrin tests (Moore and Stein, 1948) and by radioactivity determinations using a well-type NaI (Tl) crystal scintillator connected to a suitable scaling unit.

Nonradioactive thyroxine, triiodothyronine, diiodothyronine and diiodotyrosine were obtained from usual commercial sources. Monoiodotyrosine was kindly supplied by Dr. Rosalind Pitt-Rivers. Radioactive I^{131} compounds were either prepared by us or purchased from Abbott Laboratories. Radioactive triiodothyronine was prepared both by exchange with triiodothyronine and by direct iodination of diiodothyronine, and was further purified and isolated by either paper or column chromatography. Radioiodide was purchased from Abbott Laboratories. Radioactive I^{131} diiodotyrosine was prepared by exchange. Radioactive monoiodotyrosine was obtained from the column resolution of trypsin hydrolysates (see below) of thyroid tissue from rats given NaI^{131}.

RESULTS

1. *Recovery of Known Compounds.*—Table 1 contains data on the recovery of purified radioactive compounds (thyroxine, triiodothyronine, iodide, diiodotyrosine and monoiodotyrosine). These substances were added singly

TABLE 1. RECOVERY OF RADIOACTIVITY AFTER ADDITION OF RADIOACTIVE COMPOUNDS TO THE COLUMN

Radioactive compound added*	Per cent of radioactivity recovered in fractions:*						
	T_4	T_3	I	T_{y2}	T_{y1}	Int.	C.R.
T_4	95	0	2	0	0	2	1
T_4	94	0	2	0	0	4	0
T_4	96	1	1	0	0	2	0
T_3	1	98	1	0	0	0	0
I	0	0	97	0	0	2	1
I	1	0	96	0	0	3	0
T_{y2}	0	0	0	98	0	1	1
T_{y2}	0	0	0	96	0	3	1
T_{y1}	0	0	0	1	95	3	2
T_{y1}	0	0	0	1	94	3	2

* Abbreviations:

T_4 = Thyroxine
T_3 = Triiodothyronine
I = Iodide
T_{y2} = Diiodotyrosine
T_{y1} = Monoiodotyrosine
C.R. = Column residual
Int. = I^{131} occurring between peaks

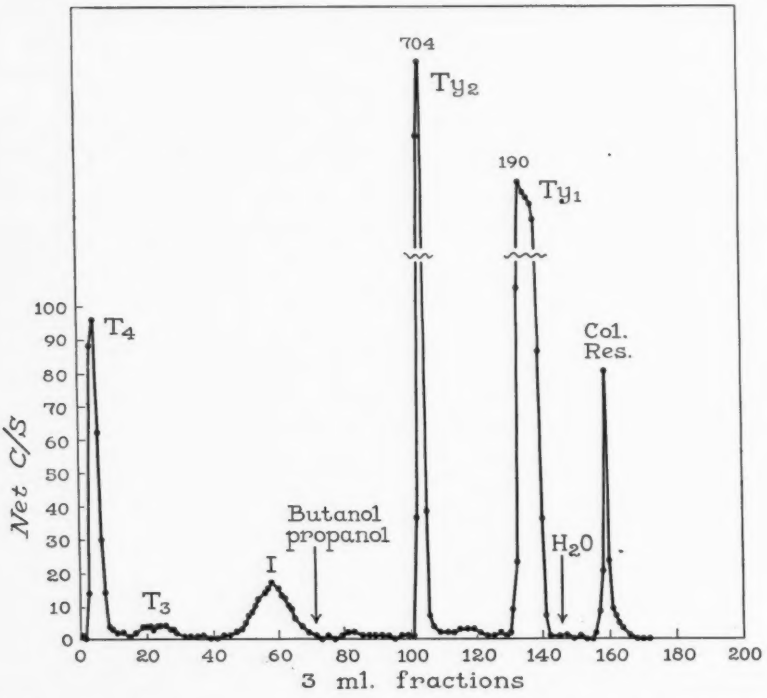

FIG. 1. Effluent diagram of 96-hour trypsin hydrolysate of gland Sc (Table 2). Abbreviations same as in Table 1 (Net C/S = net counts per second).

to the column. Over-all recoveries of radioactivity ranged from 95 to 105% and recoveries of the I^{131} in the appropriate peak area in the effluent were better than 94% in all instances. Apparently very little breakdown of these compounds occurs during the chromatographic procedure.

The position of these substances in the effluent is shown in Figure 1. The sequence of mobility of these five substances is regular and constant. In addition, diiodothyronine appears just ahead of iodide, and the conjugates (? glucuronides) of thyroxine and triiodothyronine[4] are eluted together 60 ml. ahead of diiodotyrosine.

2. *Comparison of Sodium Hydroxide and Trypsin Hydrolysis of Thyroid Tissue.*—Basic to any procedure for the quantitation of the iodinated amino acids of thyroid tissue is the method employed for hydrolysis. Although sodium hydroxide (Leland and Foster, 1932) and trypsin (Haring-

[4] Recovered labeled with I^{131} from paper chromatograms of bile from rats which had been given radiothyroxine or radiotriiodothyronine.

ton and Randall, 1931) hydrolysis of thyroid tissue have been used for many years, no chromatographic evaluation of these methods is available.

Histologically normal human thyroid tissue was obtained from euthyroid patients who were undergoing partial thyroidectomy for carcinoma or adenoma of the thyroid gland and who had received 300 to 500 μc. of I^{131} 48 hours previous to the operative procedure. Fifty to one hundred milligrams of tissue was ground in a glass homogenizer and hydrolyzed. Two N sodium hydroxide (2–5 ml.) at 95° to 100° C. for 15 to 20 hours, and in one instance, 1 ml. of an 8% barium hydroxide solution at 95° to 100° C. for 6 hours were used for hydrolysis. After cooling, concentrated hydrochloric acid was added until the hydrolysate was just pink to phenolphthalein. The solution was dried in a stream of nitrogen at room temperature and after the addition of 1 drop of concentrated hydrochloric acid the residue was extracted with successive 0.4 ml. portions of n-butanol, ethanol and 0.5 N sodium hydroxide. An aliquot (usually 0.8 ml.) of the pooled extracts was added to the column for resolution.

The second type of hydrolytic agent was trypsin. In this case the tissue was ground in 0.5 ml. of a 0.1 N ammonia, ammonium chloride buffer at pH 8.4. Hydrolysis was initiated by the addition of 0.5 ml. of buffer, 2 mg. of trypsin (Fisher) and 40 mg. of urea (per 100 mg. tissue), and was carried out for 16 to 96 hours at 36° to 40° C. in a stoppered tube. During this time further additions of buffer (totaling 0.5 ml.) and trypsin (totaling 4 mg.) were made as the hydrolysis progressed. No toluene was added to the mixture. At the end of the appropriate time, the hydrolysate was evaporated, extracted and subjected to column resolution as outlined for the sodium hydroxide hydrolysis except that it was not neutralized with HCl before evaporation.

Table 2 contains data relative to the method of hydrolysis. In the cases of the sodium hydroxide hydrolyses, the barium hydroxide hydrolysis, the trypsin hydrolysis of a trichloroacetic acid precipitate (Fig. 2) and the trypsin hydrolysis in the presence of toluene and sodium thiosulfate, note the high proportion of the radioactivity in the column residual fraction and the presence of appreciable radioactivity in an area just ahead of the di-

TABLE 2. COMPARISON OF HYDROLYSIS PROCEDURES

Gland	Hydrolysis procedure		Per cent of total effluent radioactivity in fractions:							
	Agent	Duration, hr.	T_4	T_3*	I	Pre-T_{y^2}	T_{y^2}	T_{y^1}	Int.	C.R.
W†	NaOH, 2N, 2 ml.	17	5	0	11	6	29	12	6	31
McG	NaOH, 2N, 3 ml.	15	7	0	23	8	22	10	8	23
A†	NaOH, 2N, 5 ml.	19	5	0	16	8	34	20	5	11
C†	NaOH, 2N, 5 ml.	20	6	0	19	12	29	16	5	13
F	NaOH, 2N, 2 ml.	20	6	0	8	9	39	14	7	19
R†	$Ba(OH)_2$, 8%, 1 ml.	6	2	0	12	4	15	9	10	49
F	Trypsin	48	9	0	4	4	50	23	5	6
S	Trypsin	96	8	2	6	0	49	27	2	7
K†	Trypsin	48	4	0	6	10	17	27	8	28
K‡	Trypsin	94	9	0	10	8	16	25	4	29
K	Trypsin	16	18	0	6	2	27	22	5	20
K	Trypsin	48	20	0	6	2	36	23	7	6
K	Trypsin	91	26	3	8	1	36	21	2	2
Ko	Trypsin	96	11	1	3	0	45	33	2	5
Sc	Trypsin	96	11	2	7	0	38	35	3	5

Abbreviations same as in Table 1 (Pre-T_{y^2} = Prediiodotyrosine area).
* Peak not well defined except when quantitated.
† Trichloroacetic acid precipitate.
‡ One drop of toluene and one crystal of sodium thiosulfate added to gland homogenate before hydrolysis.

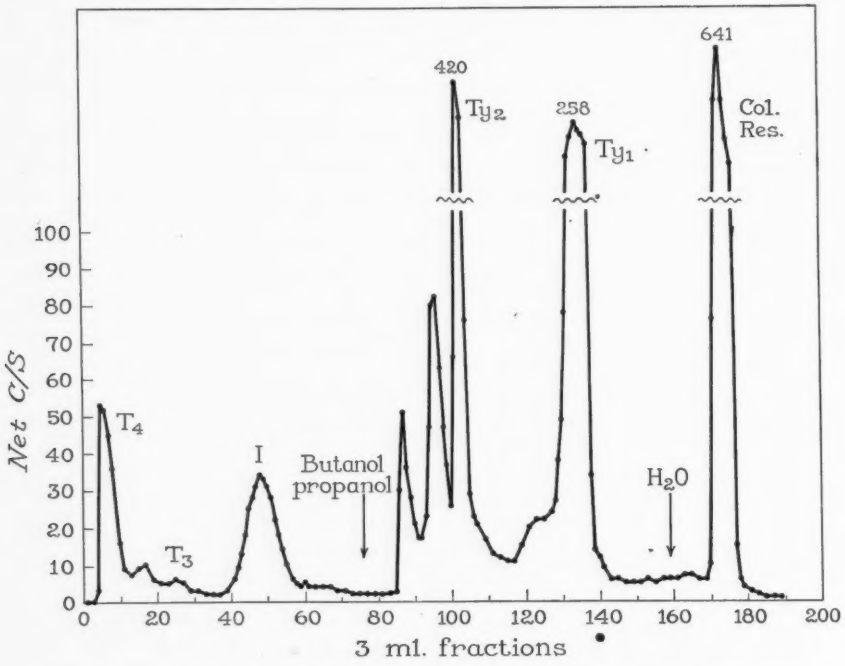

FIG. 2. Effluent diagram of 48-hour trypsin hydrolysate of trichloroacetic acid precipitate of gland K† (Table 2). Abbreviations same as in Figure 1.

iodotyrosine peak (prediiodotyrosine area). The high proportion of activity in these areas is thought to represent incompletely hydrolyzed thyroglobulin.

Support for this concept is gained (Table 3) from an experiment which shows the effect of base rehydrolysis on the column residual fraction of a trypsin hydrolysis. The radioactivity is redistributed among the thyronines, tyrosines and iodide in proportions similar to the results of base

TABLE 3. RECOVERY AND COMPOSITION OF COLUMN RESIDUAL FRACTION

Radioactivity added	Per cent of radioactivity recovered in fractions:						
	T_4	T_3	I	T_{y2}	T_{y1}	Int.	C.R.
Column residual	0	0	0	1	1	9	88
Column residual	0	0	3	2	3	4	88
Rehydrolyzed* column residual	7	1	25	19	10	14	25

Abbreviations same as in Table 1.
* Rehydrolyzed for 20 hours with 2 ml. 2N NaOH.

hydrolysis of whole thyroid tissue. In addition, reference to Table 2 shows that hydrolyses yielding high proportions of the radioactivity in the prediiodotyrosine area and column residual fraction have a lower proportion of thyroxine and diiodotyrosine. Extending the time of trypsin hydrolysis to 4 days (Table 2) improves the delineation of the triiodothyronine peak and decreases the proportion of radioactivity in the prediiodotyrosine and column residual areas. Because of the apparent incompleteness and the large amount of inorganic iodide after sodium hydroxide hydrolysis, it is our opinion that this hydrolytic agent is inferior to trypsin.

3. *Evaluation of the Combined Trypsin Hydrolysis and Kieselguhr Column Procedures.*—The trypsin hydrolysis, evaporation, extraction and column procedures were further evaluated for iodine exchange, compound breakdown and accuracy of column resolution by adding chromatographically purified radioactive thyroxine, triiodothyronine, iodide, diiodotyrosine or monoiodotyrosine to nonradioactive thyroid tissue. Table 4 contains data from these experiments. It can be seen that there was little if any exchange of radioactive iodide. The tyrosines were recoverable to 90% and the thyronines to 73% for thyroxine and 84% for triiodothyronine. It is apparent that some breakdown of the thyronines mainly to iodide (17 and 5%) occurred. It is also apparent that some of the triiodothyronine radioactivity appeared in the thyroxine area associated with the solvent front (7%). There was no apparent breakdown of thyroxine to triiodothyronine.

I^{131} losses during trypsin hydrolysis, evaporation, extraction and column resolution have been calculated using glands taken from patients who received a radioiodine tracer before the tissue was obtained. The over-all re-

TABLE 4. RECOVERY OF RADIOACTIVITY AFTER ADDITION OF RADIOACTIVE COMPOUNDS TO THYROID TISSUE*

Radioactive compound added	Per cent of radioactivity recovered in fractions:						
	T_4	T_3	I	T_{y2}	T_{y1}	Int.	C.R.
T_4	72	0	18	1	1	7	1
T_4	73	0	15	0	0	9	3
T_3	7	82	5	2	0	2	3
T_3	6	86	4	1	0	2	2
I	1	0	98	0	0	1	1
I	1	0	97	0	0	3	0
T_{y2}	0	0	3	92	1	3	1
T_{y2}	0	0	4	90	1	3	1
T_{y1}	0	0	5	0	90	3	2
T_{y1}	0	0	4	1	90	1	3

Abbreviations same as in Table 1.

* Compounds were added singly to nonradioactive tissue which was then subjected to grinding, hydrolysis (trypsin), evaporation, extraction and resolution on the column.

covery of radioactivity present at the start of tissue homogenization was usually better than 95%. There was only a 2 or 3% loss during the hydrolysis and drying procedure. The extraction recovered 98% of the total radioactivity available. A chromatographic examination of the radioactive iodine remaining in the dried residue after extraction showed it to be made up mostly of the column residual fraction (80%). The sum of the column effluent radioactivity varied usually from 95 to 105% of the added radioactivity.

The homogeneity and identity of each peak area of the effluent from column separations of trypsin hydrolysates of radioactive thyroid tissue were tested by rechromatographing aliquots of these peaks on paper. Material for rechromatography was obtained from the effluent of the column procedure on rat or Graves' disease thyroid hydrolysates. The thyronines and iodide were rerun on paper using a 4:1 normal butanol-dioxane mixture saturated with 2N ammonium hydroxide (Gross and associates, 1950). The tyrosines and column residual fractions were rerun with a mixture of lutidine, 125 ml., and water, 44 ml., in an atmosphere of ammonia. Appropriate exposure by the paper of No-Screen X-ray film allowed localization of the radioactivity. In some instances areas of radioactivity on the paper were cut out and counted. Identification of these areas of radioactivity was achieved by running an appropriate nonradioactive compound simultaneously with the radioactive aliquot in question and development of this nonradioactive compound with diazo reagent (Albright and associates, 1953) in the case of the thyronines, palladium chloride for iodide (Gross and Leblond, 1951) and ninhydrin (Consden and associates, 1944) in the case of the tyrosines. Superimposition of the colored spot on the paper on the radioactive spot on the roentgenographic film allowed identification.

When the radioactive thyroxine peak of the hydrolysate of rat or human thyroid tissue was rerun, most of the activity was identified as thyroxine. The first two fractions of this peak contained another area of I^{131} at the solvent front distinct from thyroxine, triiodothyronine or iodide. This spot amounted to 20% of the radioactivity of the first 3-ml. fraction of the thyroxine peak and much less of the second 3-ml. fraction. Its identity is unknown. This unknown radioactivity amounted to less than 10% of the total thyroxine peak. Reruns of the fractions from the latter half of the thyroxine peak yielded only thyroxine.

When the radioactive triiodothyronine and iodide peaks of a hydrolysate of a human thyroid gland were rerun, they were found to consist of single radioactive spots superimposable respectively on the colored spots of carrier nonradioactive triiodothyronine and iodide.

Direct application without evaporation of diiodotyrosine radioactivity from hydrolysates of human thyroids yielded two radioactive spots. The

more intensely radioactive spot (70% of the total radioactivity) corresponded to the diiodotyrosine marker. The other, just ahead of diiodotyrosine (23% of the activity) was not monoiodotyrosine, or iodide. On some runs to which carrier diiodotyrosine was added, there was also a ninhydrin positive spot corresponding to this radioactivity. When the radioactive diiodotyrosine from the same source as above was evaporated before application to the paper, there was an increase in this unknown spot to 47% of the radioactivity on the paper. In spite of the fact that reruns of rat thyroid hydrolysate diiodotyrosine yielded a single spot only, we believe that this second spot may be a breakdown product of diiodotyrosine formed during evaporation or paper development.

In most of the column chromatograms, a very low peak of radioactivity was noticed following the diiodotyrosine peak. When this radioactivity from a column run of a rat gland hydrolysate was rerun on paper, the radioactive spot so produced was superimposable on the ninhydrin color of carrier diiodotyrosine.

Radioactive monoiodotyrosine of a hydrolysate of a human gland was rerun on paper and yielded a single major spot which was superimposable on the colored spot of carrier monoiodotyrosine. A very faint unknown spot of radioactivity was also seen at the solvent front. Occasionally a split peak of monoiodotyrosine radioactivity was noted in column effluents. Reruns of each of these parts on the column with carrier monoiodotyrosine yielded a single peak of radioactivity which was identical with the peak of ninhydrin positive carrier (Moore and Stein, 1948).

If the column residual fraction was applied directly to a paper and rerun with lutidine and water, three main bands of radioactivity were developable, one of which remained at the origin. Several other weaker bands were also seen. Reruns of the column residual fraction of the column indicated that none of these three main bands was any known compound (Table 3). Other than the interpretation that they may be, in part, polypeptides, their identity remains unknown.

SUMMARY

A kieselguhr column chromatographic procedure which is applicable to the separation of thyroxine, triiodothyronine, iodide, diiodotyrosine and monoiodotyrosine has been described and evaluated. When added to the column in pure state, the recovery of each of these compounds in its respective peak area is better than 94 per cent.

This chromatographic procedure was used to evaluate the hydrolysis of radioactive (I^{131}) thyroid tissue. A trypsin method was shown to provide more complete hydrolysis with a similar or smaller risk of degradation of the liberated amino acids than the standard sodium hydroxide method.

Chromatographically pure radiothyroxine, triiodothyronine, iodide, diiodotyrosine and monoiodotyrosine were added to nonradioactive thyroid tissue which was subsequently homogenized, hydrolyzed with trypsin, evaporated, extracted and subjected to this column procedure. Thyroxine and triiodothyronine were recoverable to 73 and 84 per cent respectively and the tyrosines to better than 90 per cent. No appreciable iodine exchange occurred.

The radioactive peaks obtained in chromatographic effluents from runs of trypsin hydrolysates of radioactive thyroid tissue were tested as to identity and homogeneity by paper chromatography. The presence of thyroxine, triiodothyronine, iodide, diiodotyrosine, and monoiodotyrosine in human gland hydrolysates was confirmed. In each of these specific peak areas, no quantitatively serious contamination was found.

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THE INFLUENCE OF AGE AND SEX ON THE SUCCINOXIDASE ACTIVITY OF THE ADRENAL GLAND OF THE RAT[1]

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THERE are indications of a parallelism between oxidative processes in the adrenal cortex and the synthesis of corticosteroids (Carpenter, McLeod and Reiss, 1946; Tepperman, 1950; Perry and Cumming, 1952). We have undertaken an investigation of the oxidative metabolism of the adrenal gland of the rat, under various physiological conditions, and for the experiments reported here have chosen the succinoxidase system for study because of its obvious importance in respiratory oxidations. Furthermore, the activity of this system is such that it can be measured in small amounts of tisssue.

During the investigation it became apparent that there was a pronounced difference in adrenal succinoxidase activity between male and female rats. There is evidence that the gonads exert a marked influence upon adrenocortical morphology and function, although the nature of this influence is not clear (Roberts and Szego, 1953). Both activation and inhibition of adrenocortical function by estrogens and by androgens have been reported. In general the estrogens appear to activate cortical function while androgens act as inhibitors (Tepperman, Engel and Long, 1943; Parkes, 1945; Emmens and Parkes, 1947). Because of the connection between gonadal function and age, the present work is concerned with the influence of both age and sex on the succinoxidase activity of the adrenal gland of the rat.

METHODS

Rats of the Wistar strain bred in the Department of Physiology were used. Animals of both sexes were chosen from each of three age groups: 4 to 5, 7.5 to 9, and 17 to 19 weeks. The animals were kept in groups of between 7 and 10 to a cage and fed fox breeder cubes (Master Feeds) and water *ad libitum*. They were killed by dislocation of the cervical cord and were allowed to bleed through the severed aorta. The adrenal glands were removed immediately, trimmed of fat, and weighed on a torsion balance. They were bisected and placed in a glass Potter-Elvehjem homogenizer tube. Cold glass-

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distilled water was added in an amount necessary to make a 5% homogenate and the glands were homogenized for three minutes.

Succinoxidase activity was measured manometrically by the method of Schneider and Potter (1943), under conditions in which, presumably, the limiting factor of the overall reaction is the activity of the succinic dehydrogenase. Each vessel contained: 1.0 ml. of 0.1 M phosphate buffer (pH 7.4), 0.4 ml. of 10^{-4} M cytochrome c (Sigma Chemical Co.), 0.3 ml. of 4×10^{-3} M $AlCl_3$, 0.3 ml. of 0.5 M sodium succinate (pH 7.4), 0.4 to 0.6 ml. of a 5% adrenal gland homogenate, and distilled water to make up 3.0 ml. The center well contained 0.2 ml. of 10% KOH and a folded piece of filter paper 1 inch square. All vessels were kept on cracked ice during the preparation period which lasted between 45 and 60 minutes. After equilibration for 15 minutes at 38° C. (with air as the gaseous phase) readings were taken at intervals of 10 minutes, for one hour. The vessels were then removed from the water bath. Because of the appearance of coarse agglutination particles during the incubation period, each sample was again homogenized, and an aliquot was taken for nitrogen determination by the micro-Kjeldahl method.

In the four week old animals the glands from 3 rats were pooled because 20 to 30 mgs. of tissue were required for each determination. In the eight week old group the glands from 2 animals were pooled. In the older animals each determination was done with the adrenals of one rat.

The succinoxidase activity is expressed as total oxygen uptake in terms of microliters of oxygen per 1 mg. of adrenal nitrogen during the first hour. The % adrenal nitrogen in both sexes at the three age levels studied is remarkably constant; therefore, the results have not been expressed in terms of wet weight of gland. Because both adrenal gland size and body weight of the two sexes at various ages differ, the results have also been expressed as microliters of oxygen per pair of glands, and per 100 gm. of body weight, per hour.

The results were analyzed statistically by means of Fisher's "t" test.

RESULTS

At four weeks of age the succinoxidase activity was found to be approximately 21% higher in the adrenal glands of the males ($p<0.01$). Neither the adrenal weights nor the body weights differed in the two sexes and, therefore, the oxygen uptake corresponding to the total amount of gland, and to 100 gm. of body weight, was also approximately 23% higher in the males than in the females (Table 1). At this age all the females had closed vaginas and about 50% of the males had undescended testes.

At 7.5 to 9 weeks of age, the adrenal succinoxidase activity in the males was 82% higher than in the females ($p<0.01$). However, since the adrenal glands of the males were 30% lighter than those of the females the oxygen uptake by the total gland tissue was only 20% higher in the males, a difference not statistically significant. The average body weight of the males was 30% greater than that of the females; therefore the oxygen uptake per 100 gm. of body weight was similar in both groups (Table 1).

At 17 to 19 weeks of age the adrenal succinoxidase activity in relation to nitrogen content was 79% higher in the males than in the females ($p<0.01$). However, since the adrenals of the males were 24% lighter than

TABLE 1. SUCCINOXIDASE ACTIVITY OF ADRENAL GLANDS OF MALE AND FEMALE RATS AT 4, 8 AND 18 WEEKS OF AGE
(Average values + or − the standard error of the mean)

Sex and age in weeks	No. of animals (No. of observations)	Body wt., gm.	Adrenal gland			μl. O_2 in 1st. hour		
			Wt., mgs.	wt., mgs./100 gm. body weight	N %	Per mg. adrenal N	Per pair of glands	Per 100 gm. body wt.
M 4–5	30 (10)	75±2	18.0±0.4	24.0±0.7	2.57±0.03	256±2	118±4	158±2
F 4–5	30 (10)	74±3	17.8±0.3	24.0±0.7	2.55±0.03	212±2	94±3	128±2
M 7.5–9	14 (7)	198±8	26.1±0.9	13.2±0.6	2.55±0.03	308±15	203±8	103±3
F 7.5–9	14 (7)	152±3	37.8±1.8	24.8±1.0	2.59±0.04	169±9	168±16	109±9
M 17–19	7 (7)	350±10	33.2±1.5	9.5±0.4	2.23±0.12	265±18	197±20	56±5
F 17–19	7 (7)	200±7	43.6±3.0	21.8±1.4	2.50±0.06	148±7	164±21	82±10

those of the females the oxygen uptake corresponding to the total gland tissue was only 20% higher in the males, a difference not statistically significant. The average body weight was 75% higher in the males than in the females (Table 1); therefore, the oxygen uptake per 100 gm. of body weight was 30% lower in the males. This difference does not quite meet the requirements of statistical significance ($0.1>p>0.05$) (Table 1).

The rates of oxygen uptake corresponding to the six groups of animals are shown graphically in Figure 1. It can be seen that the rates decline from the first 10 minute period onwards in all groups.

DISCUSSION

The results show that the adrenal gland of the male rat has a considerably higher succinoxidase activity per unit of nitrogen content than that of the female at all age levels studied. That this difference is related to gonadal function is supported by the fact that the magnitude of the difference is smallest (21%) in the sexually immature groups (Fig. 2).

Since whole adrenal glands were used, the question arises as to whether the succinoxidase activity measured represents the activity of both cortex and medulla or mainly that of the cortex. The relative volumes of cortex and medulla in the adrenals of the rat have been shown to bear a ratio of 10:1 or greater, (Donaldson, 1919; Jackson, 1919) the ratio being bigger in the female than in the male. The actual amount of medullary tissue in the glands of the two sexes is the same so that the difference in adrenal gland size is due to a difference in the amount of cortical tissue. Furthermore, Sourkes and Heneage (1952) have shown that the rate of oxidation of succinate is about twice as great in cortical as in medullary tissue. This

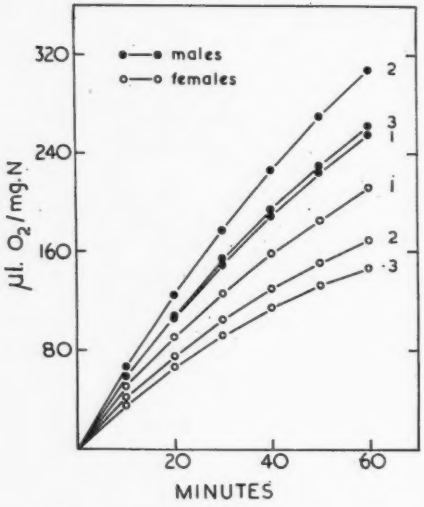

FIG. 1. Rates of O_2 uptake by the succinoxidase system of adrenal glands of male and female rats of different ages. 1) rats aged 4 weeks; 2) rats aged 8 weeks; 3) rats aged 18 weeks.

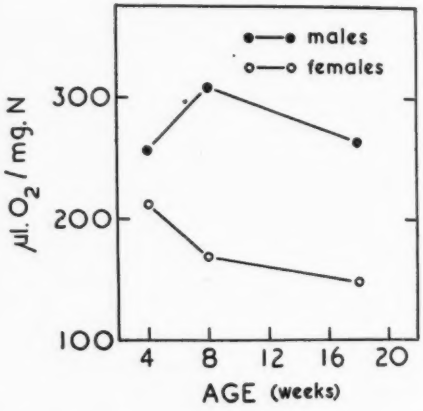

FIG. 2. Influence of age and sex on the succinoxidase activity of adrenal glands of rats.

means, in all probability, that the differences observed in the present study are due mainly to differences in cortical tissue activity.

While the conditions used for measuring succinoxidase activity are known to provide a measure of the succinic dehydrogenase activity when applied to preparations such as liver and muscle, it remains to be shown whether this is the case in the adrenal preparations. Factors other than the dehydrogenase might presumably affect the rate of the overall reaction in particular cases. Therefore, for the present, the results must be considered to represent overall succinoxidase activity rather than a selective evaluation of the dehydrogenase activity.

It will be seen from the progress curves of oxygen uptake in Figure 1 that the falling off in the rate is more pronounced in the case of preparations from female animals than from male animals. A study of the slopes of the individual curves has shown that this difference between the sexes is less marked at four weeks of age than in the older animals. An explanation of the progressive falling off of the velocity in all preparations of the succinoxidase from adrenal glands, and of the interesting sex difference in this respect must await a more detailed study of these preparations.

The physiological significance of the marked difference in succinoxidase

activity of male and female rat adrenals will not become apparent until a better understanding is gained of the manner in which oxidative enzyme activity is related to the specific functions of the gland. There is some evidence that conditions known to increase secretion of cortical hormones, like administration of ACTH or cold exposure, increase the oxidative metabolism of the adrenals while cortisone administration decreases it (Carpenter, McLeod and Reiss, 1946; Perry and Cumming, 1952). However, the evidence so far available does not seem enough to conclude that under all conditions hormone synthesis and oxidative metabolism run parallel. Whatever the significance of these findings may be, it is clear that changes in adrenal gland size, per se, should be interpreted with considerable caution. This is of particular interest in relation to the effects of gonadal secretions on adrenal function, since a great deal of the available evidence indicating that estrogens stimulate, while androgens inhibit the adrenal cortex, has been based on adrenal size measurements. Although the findings herewith reported do not necessarily exclude such a conclusion, they make it questionable, and therefore subject to revision.

SUMMARY

The succinoxidase activity of the adrenal glands of male and female rats of approximately four, eight and eighteen weeks of age has been measured in whole gland homogenates. This activity, expressed in terms of adrenal nitrogen, is significantly greater in the glands of the males than in those of the females at all ages studied. The difference is smallest (21%) at four weeks of age and greatest (80%) at eight and eighteen weeks of age.

Acknowledgment

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EFFECTS OF PINEAL EXTRACT ADMINISTRATION ON OVARY WEIGHT IN RATS[1]

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PINEALECTOMY in the immature rat causes statistically significant increases in gonadal weight (Izawa, 1926; Kitay, 1954; Simonnet *et al.*, 1951). On the other hand, the administration of pineal extract in the rat is said to produce either gonadal hypertrophy (Milco, 1941), gonadal atrophy (Engel, 1934; Moszkowska, 1951; Tsuchimoto, 1933; Wakida, 1944), or no change in gonadal weight (Hartog Jager and Heil, 1935; Hoskins, 1916; Roux, 1937; Sisson and Finney, 1920; Tomorug and Hussar, 1942; Waysbaum-Moschkowski, 1945). The last finding has been generally accepted despite the fact that all of the published data on pineal extract administration in the rat are fragmentary and inconclusive. In view of this lack of statistically significant data, the following preliminary study was undertaken.

MATERIAL AND METHODS

Powdered bovine pineal glands were supplied by the Wilson Laboratories of Chicago, Illinois. The glands, obtained from two-year-old government-inspected steers, had been removed and frozen shortly after slaughtering. When the glands had been thawed out in the laboratory, the tissue juice was extracted by pressure and mixed with four parts of cold acetone. The resulting precipitate was dried rapidly and stored at room temperature. The pineal extract used in the experiments described below was prepared by mixing the powder with distilled water in a Waring blendor. Undissolved material was removed by centrifuging and the solution was passed through a bacteriological filter. The concentration of the extract was adjusted so that 1 ml. contained the soluble material from 100 mg. of pineal powder. The preparation was kept frozen until the day of use.

Three series of female Wistar rats were given the pineal extract by daily intraperitoneal injection for fourteen days. The rats of Series 1 were started at 32 days of age and received daily injections of 0.5 ml. of pineal extract (equivalent to 50 mg. of powder). The animals in Series 2, started at 30 days, and those in Series 3, started at 45 days of age, all received 1.0 ml. daily (equivalent to 100 mg. of powder).

Control groups in each series were given equivalent volumes of distilled water. Ovary and body weights were measured in all three series at autopsy on the day following the

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last injection. Diet for all animals consisted of Purina Laboratory Chow, and water supplied *ad libitum*.

RESULTS

The mean ovary weights of the various pineal-treated and control groups are presented in Table 1. Analysis of covariance of ovary weight and body

TABLE 1. EFFECTS OF PINEAL EXTRACT ADMINISTRATION ON MEAN OVARY WEGIHT

Series	Age at onset (days)	Age at autopsy (days)	No. of rats	Treatment	Mean ovary wts. ±S.E.* (mg.)	*P*	Mean ovary wts. ±S.E.† (mg.)	*P*
1	32	47	19	Pineal	29.7±2.4	>0.05	30.3±1.0	<0.05
			19	Water	34.0±1.7		33.4±0.9	
2	30	45	21	Pineal	18.7±1.1	<0.001	18.8±1.0	<0.001
			21	Water	29.3±1.5		29.1±1.5	
3	45	60	18	Pineal	40.4±2.8	<0.05	42.7±1.6	>0.05
			18	Water	49.0±2.4		46.7±2.1	

* Mean ±Standard Error of the mean.
† Adjusted Mean ±Standard Error of the mean after correction by analysis of covariance.

weight was made for each group; the mean ovary weights, adjusted for variation in body weights, also are shown in the table. Diminution of ovary weight in the pineal-treated groups was observed in all three series. The observed decrease in mean ovary weight in Series 1 is not statistically significant of itself. However, when variation in body weight is taken into account, through analysis of covariance, the difference proves to be significant at the 5% level of confidence. The ovaries of the rats in this series did not differ in appearance from those of control animals. The mean ovary weight of the pineal-treated group in Series 2 was 36% less than that of the control group. This difference is significant at the 0.1% level, and the significance is unchanged by analysis of covariance. In Series 2, 19 of the 21 control rats had hemorrhagic ovarian follicles, whereas only 3 pineal-treated animals showed such findings. This difference also is significant at the 0.1% level of confidence (Table 2). The decrease in mean ovary weight in the pineal-treated rats of Series 3 is significant at the 5% level. However, this difference is not significant when variation in body weight is taken into account through analysis of covariance. The ovaries of the animals in

TABLE 2. CONDITION OF OVARIES OF THE RATS IN SERIES 2

Findings	Pineal	Water	Totals
Hemorrhagic follicles	3	19	22
Infantile	18	2	20
Totals	21	21	42

(Chi-square = 21.4; $P \leq 0.001$.)

this series did not differ in appearance from those of the control animals. The body weights of the pineal-treated and control groups did not differ significantly in any series.

DISCUSSION

The results in Series 2 demonstrate that two weeks' daily administration of 1.0 ml. of pineal extract produces marked ovarian atrophy in immature animals. This atrophy was not influenced by variation in body weight. The treatment also inhibited the formation of corpora hemorrhagica. On the other hand, the indication that ovarian atrophy was produced by daily injection of 0.5 ml. of pineal extract in Series 1 has only borderline significance when examined statistically. The variation in significance between the observed difference and the difference obtained by correcting for body weight may be explained by the fact that the animals, though of the same age were a mixture of two lots with varying body weights.

The effectiveness of daily injection of 1.0 ml. of extract as compared with the relative ineffectiveness of a dose of 0.5 ml. suggests a method of assaying the potency of pineal extracts. Ovarian atrophy, inhibition of follicular changes, or both of these might be used as the criterion in such an assay.

The results in Series 3 were of questionable statistical significance; they suggest that pineal extract may not influence ovarian weight in animals that are treated when sexually mature.

SUMMARY

Daily administration of pineal extract for fourteen days produces ovarian atrophy in the immature rat.

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THE SITE OF FOLLICLE STIMULATING AND LUTEINISING HORMONE PRODUCTION IN THE RAT PITUITARY

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IN A previous publication (Purves and Griesbach, 1951) the presence of two distinct kinds of basophil cells in the anterior pituitary of the rat was reported. Evidence was presented that one of these kinds was exclusively concerned with the production of thyrotrophic hormone, while the other was exclusively gonadotrophic in function. It was considered that the latter cells were responsible for the secretion of the follicle stimulating hormone, but the observations made at the time were inadequate to establish the site of production of the luteinising hormone. More recent observations have shown that the gonadotrophic basophils of the rat pituitary may be further subdivided into two specific types. One type has a peripheral distribution in the anterior lobe, and under most circumstances, gives a strong glycoprotein reaction with the periodic acid-Schiff procedure for the demonstration of glycoprotein. In the interior of the anterior lobe, basophil cells of rounded contours which are quite distinct from the thyrotrophs may be demonstrated under favourable circumstances (Purves and Griesbach, 1952; Siperstein, Nichols, Griesbach and Chaikoff, 1954). The intensity of the glycoprotein reaction in these cells is, under most circumstances, much weaker than that in the peripheral gonadotrophs, which accounts for the presence of these cells in normal pituitaries being overlooked in the earlier investigations. Since both types react strongly in accordance with variations in gonadotrophin secretion, both types are presumed to be gonadotrophic in function. If the two types can be established as being functionally distinct, then it would be probable that one of them is exclusively responsible for follicle stimulating hormone, and the other for luteinising hormone secretion.

The functional differentiation of the two gonadotrophic types is difficult to achieve since, under most circumstances, the activities of the two types of cells run roughly parallel. It is, therefore, necessary to produce an artificial condition in which a differential effect on the secretion of the two gonadotrophic hormones may be observed. Greep and Jones (1950) have

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shown that treatment of adult female rats with appropriate doses of testosterone leads to an increased potency of the pituitary gland in follicle stimulating hormone, and a reduction and eventual disappearance of luteinising hormone. The effects of testosterone injections on the glycoprotein content of the gonadotrophs of the adult female rat pituitary have therefore been investigated. Results indicate that the peripheral gonadotrophs are follicle stimulating in function, while the central gonadotrophs are probably luteinising.

MATERIALS AND METHODS

Adult female rats, three months of age, were injected with testosterone propionate ("Perandren" Ciba, 250 μg. per day) for periods up to 28 days. The rats also received injections of 5 μg. of L-thyroxine per day. The thyroxine was given for the purpose of suppressing the glycoprotein content and, therefore, the visibility of thyrotrophs, thus enabling a clearer picture of the centrally situated gonadotrophs to be obtained. For the purpose of comparison, pituitary glands have been obtained from immature rats, from mature female rats in cyclical activity, and from pregnant female rats. Some of these animals received injections of L-thyroxine, while others were untreated.

Histological Methods. For histological examination, the pituitaries were removed immediately after death and fixed in formol-sublimate (1:9). Sections cut at 5 μ were subjected to the periodic acid-Schiff procedure (PAS) as previously described (Purves and Griesbach, 1951). A faint nuclear staining was obtained by staining for two to five seconds in Harris' haematoxylin, diluted one in ten with 1% acetic acid.

RESULTS

The thyroxine treatment in all cases caused the disappearance of the angular thyrotrophic basophils, and the remaining basophils were round or oval in outline. Control experiments showed that the round or oval basophils were equally well visualized in animals not treated with thyroxine, being distinguished by their shape from the characteristically angular thyrotrophs. In the doses used in these experiments thyroxine, therefore, totally inhibited the thyrotrophs without showing any apparent effect on the non-thyrotrophic basophils.

In the immature female rats rounded basophil cells with distinct glycoprotein containing granules are present throughout the anterior lobe of the pituitary gland (Figs. 1 and 2). The peripherally situated gonadotrophs are in general distinguished by a coarser granulation and more strongly positive glycoprotein reaction. The centrally situated gonadotrophs have a much finer granulation, and the intensity of the glycoprotein reaction is weaker, so that the colour obtained by the PAS reaction is pink rather than red. The centrally situated gonadotrophs are on the whole larger and rounder than the peripherally situated type. They are normally seen in small aggregates only, and they do not show the same affinity for the larger blood vessels that is often a striking characteristic of the peripheral

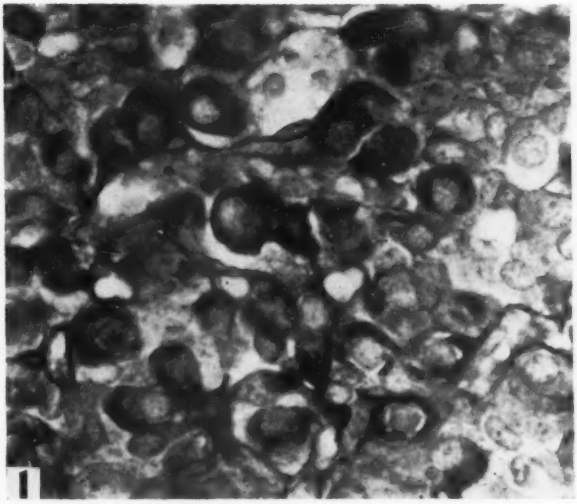

FIG. 1. Pituitary of twenty-five day old female rat. Periphery of anterior lobe, showing gonadotrophs containing coarse dark granules. Perivascular arrangement of cells. PAS, haematoxylin. ×675.

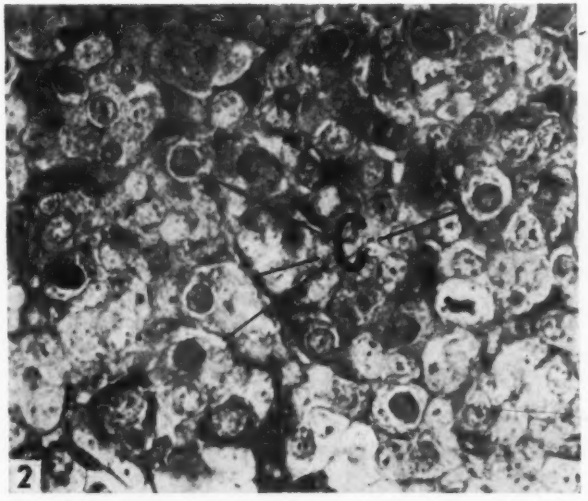

FIG. 2. Centre of same section as in Fig. 1. Large round cells with fine granulation (C). Nuclei of these cells are denser than nuclei of most other cells. PAS, haematoxylin. ×675.

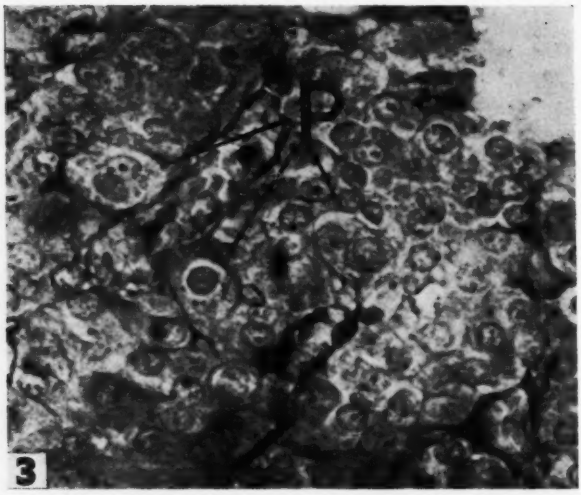

FIG. 3. Pituitary of fifty-six day old female rat. Periphery of anterior lobe. Gonadotrophs (P) appear degranulated. PAS, haematoxylin. ×675.

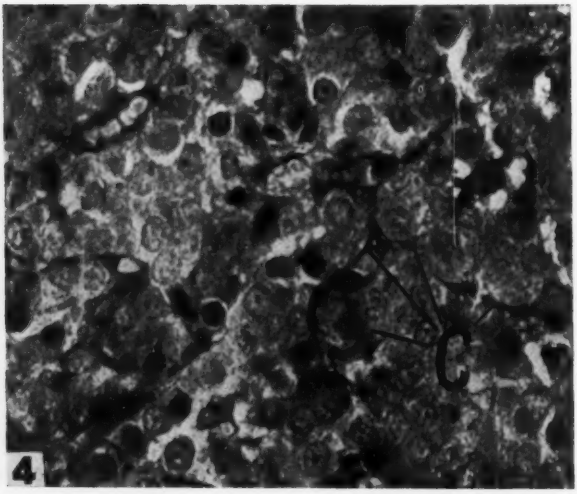

FIG. 4. Centre of same section as in Fig. 3. In the right half of the section, round pale cells (C) are seen in contact with capillary. PAS, haematoxylin. ×675.

type. In general, the golgi body, visible as a negative image in PAS preparations, is more conspicuous in the centrally situated cells than in the peripheral gonadotrophs.

In the adult female, during cyclic estrous activity, gonadotrophic basophils are infrequent and difficult to identify and study. Apparently the cyclic discharge of gonadotrophic hormones with recurrent estrous cycles has so lowered the glycoprotein content that identification of the gonadotrophic cells is, in most instances, impossible (Figs. 3 and 4). During pregnancy, when the estrous cycle is interrupted, large numbers of basophils are demonstrable in the female rat pituitary by PAS and by other staining methods. The thyrotrophs and gonadotrophs share in this marked basophil activation during pregnancy. Pre-treatment with thyroxine totally inhibits the thyrotrophs, and in such animals the presence of gonadotrophs of both the peripheral and central types is easily demonstrated. The characteristic larger size, rounder contour, finer granulation and more conspicuous golgi body of the central gonadotrophs are especially marked in the pregnant female rat pituitary (Figs. 5 and 6).

The administration of testosterone to the mature non-pregnant female rat resulted after 10 days in a marked increase in the numbers of both central and peripheral types of gonadotrophs. After 21 days, a differential action of the testosterone on the two types of gonadotroph was apparent. The central gonadotrophs had regressed and were represented by small, shrunken cells, with pyknotic nuclei. The glycoprotein reaction in these cells was either faint or absent. On the other hand the peripheral gonadotrophs, although small, were full of coarse granules giving an intense glycoprotein reaction. After 28 days the regressive changes in the central gonadotrophs had proceeded so far that these cells could no longer be visualised. The peripheral gonadotrophs, on the other hand, presented the same appearance as after treatment for 21 days, being small inactive looking cells with pronounced glycoprotein storage in the cytoplasm (Figs. 7 and 8).

Discussion. Greep and Jones (1950) have investigated the effect of testosterone injections on the gonadotrophin potency of the female rat pituitary. They found that small doses of testosterone led to a marked increase in the gonadotrophic potency of the pituitary as determined by assay. As the dosage of testosterone was increased, the hormone content was depressed. At the highest level (500 μg. testosterone propionate per day) gonadotrophin potency was still present, but the response was purely follicle stimulating. The complete absence of luteinising hormone was made evident by the fact that the follicular stimulation in the hypophysectomised recipient rats was not accompanied by any estrogen production and the uteri of the recipient rats were, in consequence, completely un-

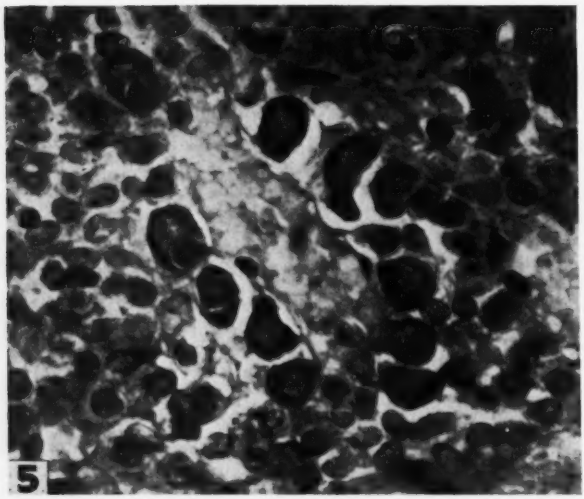

FIG. 5. Pituitary of pregnant rat, three months old. Periphery of anterior lobe. Coarsely granulated gonadotrophs may be seen round a large portal vessel. Negative Golgi images visible. PAS, haematoxylin. ×675.

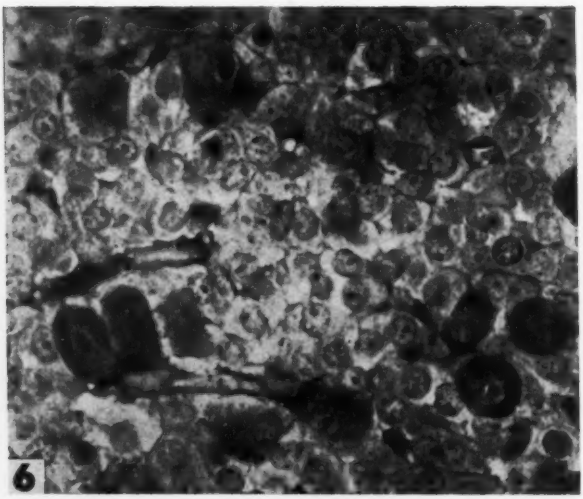

FIG. 6. Pituitary of pregnant rat, three months old. Centre of anterior lobe shows finely granulated large gonadotrophs with conspicuous Golgi image. PAS, haematoxylin. ×675.

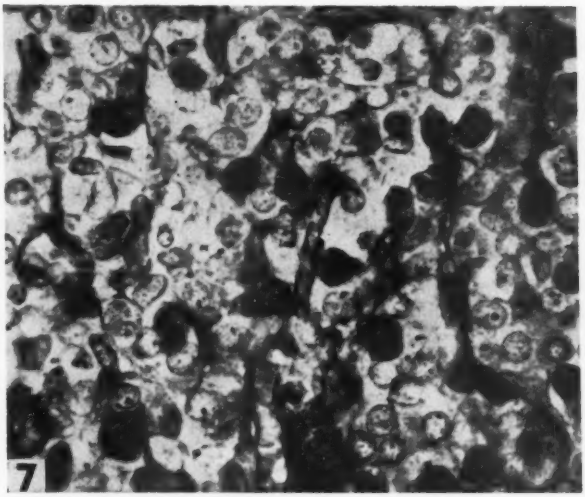

FIG. 7. Pituitary of adult female rat after twenty-eight days treatment with testosterone propionate. Periphery of anterior lobe, showing shrunken, coarsely granulated gonadotrophs along blood vessels. PAS, haematoxylin. ×675.

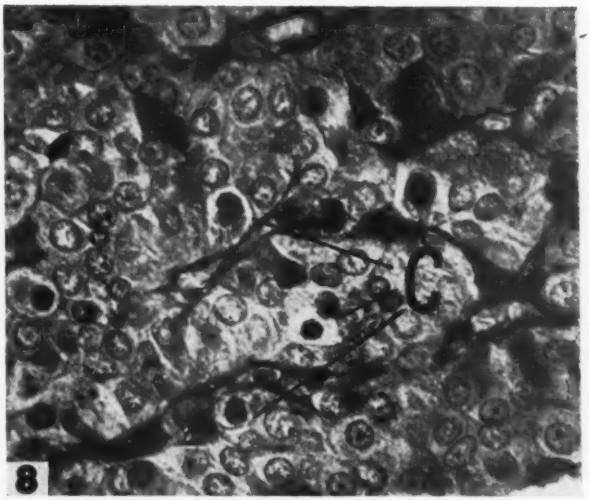

FIG. 8. Same section as in Fig. 7. Centre of anterior lobe. Small pale cells (C) with pyknotic nuclei and partly disrupted cytoplasm may be seen along capillaries. One conspicuous angular thyrotroph is visible. PAS, haematoxylin. ×675.

stimulated. In our cytological observations a differential effect of testosterone on peripheral and central gonadotrophs was obtained, such that glycoprotein storage was promoted in the peripheral type of gonadotroph, while the central type was completely inhibited. We have not controlled the complete absence of luteinising hormone from the pituitaries of our testosterone treated rats by assays on hypophysectomised recipients, but simple assays, using immature female rats as test animals, showed an increased follicle stimulating potency and complete absence of luteinisation. We therefore consider that our results, in conjunction with the assays of Greep and Jones, indicate that the follicle stimulating hormone is produced by the peripheral type of gonadotroph, while the central type is in all probability responsible for the luteinising hormone. This view will be further supported by observations on the sequence of changes in both peripheral and central gonadotrophs following castration in the rat, which are to be presented in a subsequent paper. It has been found that in pituitary glands of high luteinising potency, the central gonadotrophs are especially numerous and rich in glycoprotein.

Siperstein *et al.* (1954) have followed the changes in the basophils of the rat pituitary from birth to maturity. They distinguished between central and peripheral gonadotrophic types, and found that in immature animals both types were prominent. An interesting observation was made concerning the changes in gonadotrophs occurring at the time of natural maturation of the female. After the first estrus, which occurred at about 42 days, both gonadotrophic cell types were found to have undergone extensive degranulation. In the majority of animals examined at the age of 42 days, degranulation of the peripheral gonadotrophic cells had already occurred, while the central cells still retained their glycoptrotein granules. This observation is, therefore, consistent with the view that discharge of the follicle stimulating hormone precedes discharge of the luteinising hormone in the production of the first estrus.

Attention has been drawn to the large size and active appearance of the central gonadotrophs in the anterior pituitary of the pregnant rat. If our identification of the central gonadotrophs as the site of luteinising hormone secretion is accepted, this appearance would indicate a continued rapid secretion of luteinising hormone during pregnancy in the rat. Everett (1947) has pointed out that the corpora lutea of pregnancy in the rat differ from those maintained by injection of luteotrophin alone, in that the lipoid content of the corpora lutea of pregnancy is considerably higher than that of the corpora lutea under the influence of luteotrophin only. Luteinising hormone administered simultaneously with luteotrophin increases the lipoid content of the corpora lutea. It is, therefore, probable that the corpora lutea of pregnancy in the rat are under the simultaneous influence of

luteotrophin and luteinising hormone. This deduction from ovarian responses harmonises with the deductions made from cytological study of the anterior pituitary.

SUMMARY

In addition to basophils concerned with thyrotrophic hormone secretion, the anterior pituitary of the rat contains two specific types of basophil cell concerned with gonadotrophic hormone secretion. The peripherally situated gonadotrophs are distinguished by a coarse cytoplasmic granulation due to glycoprotein with a high sugar content. They are considered to be responsible for the secretion of follicle stimulating hormone. The centrally situated gonadotrophs have finer granules and the glycoprotein reaction is less intense. They are considered to be responsible for the secretion of the luteinising hormone. Testosterone propionate (250 micrograms per day) has a differential effect on the gonadotrophic cell types of the adult female rat pituitary, promoting granule storage in the peripheral (FSH-producing) cells, and depressing the granule content of the central (LH-producing) cells.

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INFLUENCE OF CORTISONE ON THE MORTALITY OF X-IRRADIATED ADRENALECTOMIZED MICE[1,2]

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THE involvement of alterations in adrenal cortical function, as a part of the response of the organism to x-irradiation, has been suggested in view of the chemical and histological changes in the adrenal cortex (Englestad and Torgerson, 1939), depletion of adrenal cortical cholesterol (Dougherty and White, 1946), and histochemical and adrenal weight changes (Patt *et al.*, 1947), which have been observed following exposure to x-irradiation. Lawrence (1949), reported an increased urinary excretion of neutral 17-ketosteroids following x-irradiation of dogs. However, Duffy (1952), was unable to confirm this finding. An increased level of 17-hydroxycorticosteroids was found in the adrenal vein of x-irradiated dogs by Bowers *et al.* (1952). The involvement of the adrenal cortex in x-irradiation is further supported by the amelioration of the indirect catabolic effects of x-irradiation on the thymo-lymphatic system by adrenalectomy (Leblond and Segal, 1942; Dougherty and White, 1946). Adrenalectomy has also been found to shorten the life span and to increase the radiosensitivity following x-irradiation in mice (Cronkite and Chapman, 1950; Kaplan *et al.*, 1951) and rats (Edelmann, 1951a), whereas adrenal shielding appreciably decreased mortality of x-irradiated rats (Edelmann, 1951b). These findings suggest that adrenal cortical secretions exert a protective influence following x-irradiation.

The results of attempts to protect intact animals against the effects of x-irradiation by use of adrenal cortical steroid hormones are conflicting. Success has been reported using desoxycorticosterone acetate (Ellinger, 1947, 1948; Mirand *et al.*, 1952), cortisone (Mirand *et al.*, 1952) and whole extract of the adrenal cortex (Edelmann, 1951a). Failure of hormones to

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significantly protect x-irradiated animals has been reported using DOCA (Straube *et al.*, 1949; Graham *et al.*, 1950) and cortisone or ACTH (Smith *et al.*, 1950). Ellinger *et al.* (1952), reported an increase in the mortality of intact mice treated daily with .25 mg. cortisone after their exposure to high, but not to low x-ray doses, and suggested that the effect of x-irradiation on the adrenal cortex may consist of a stimulation with eventual exhaustion of secretion and a disturbance of the balance of the various adrenal cortical hormones in the secretions.

The hemorrhagic syndrome, vascular fragility, thrombopenia, ulcerations, and other blood coagulating defects, leading to death following exposure to γ-irradiation (Cronkite, 1950), in many ways resemble those in anaphylactoid death. Dougherty (1951), using adrenalectomized mice, demonstrated that anaphylaxis is a graded response depending partly on a quantitative relation between the doses of antigen and of the protective agent. Many of the biological effects of cortisone seem to be responses of the continuous variate type (Dougherty, 1953a). These have been accurately demonstrated for its antiphlogistic (Dougherty, 1953b) and thymoregressive properties (Santisteban, 1953, and Santisteban and Dougherty, 1953). It was conceivable that the protective effect of cortisone in x-irradiated animals may also be of a similar nature; if so, such a relationship could be established only in the absence of the animals' own supply of hormone. The demonstration of such relationships between the degree of protection and doses of hormone would make possible a quantitative analysis of the protective effect and might give an insight into the mode and time of action of the adrenal cortical secretions following x-irradiation.

This report presents the results of experiments designed to establish the existence of such relationships, as judged by the effects of graded doses of exogenous hormone on the mortality of x-irradiated adrenalectomized mice.

METHODS

A total of 249 virgin female CBA mice, 12 weeks of age, were used in this study. The animals were from our own colony and were maintained on "Purina Dog Chow" and tap water, before and during the course of the experiments. Two hundred and nineteen of these animals were exposed to 650r whole-body irradiation (LD 50/30 days, 600r). Irradiation was delivered by a Westinghouse Quadrocondex x-ray tube using the following radiation factors: 250 KV, 15 Ma, at a rate of 160 r/min. with .05 mm. Cu+1 mm. Al filter. Five animals were exposed simultaneously, placed together in a perforated lucite box ($4\frac{1}{2}'' \times 4\frac{1}{2}'' \times 1\frac{1}{4}''$) at a distance of 3″ from the cone. Calibration was done on a wax phantom placed inside the box, by use of a "Victoreen r-meter." Control and experimental animals were picked at random from among the animals thus irradiated.

Two days following irradiation, adrenalectomy was performed in a one stage translumbar operation under ether anesthesia. Twelve hours after the operation, the adrenalectomized animals were divided into five groups. Four groups were treated daily with

TABLE 1. EFFECT OF GRADED DOSES OF CORTISONE ON THE MORTALITY OF X-IRRADIATED ADRENALECTOMIZED FEMALE MICE

	650r whole body x-irradiation						Adrenex.
	Adrenalectomized*					Intact (41)	(30)
	Dose of Cortisone mg./day						
	None (32)	.005 (34)	.05 (38)	.5 (42)	1 (32)		
Group No.	I	II	III	IV	V	VI	VII
Per cent mortality	100	100	100	92.9	96.9	73.2	87.0
Slopes of mortality curves	29.30 ±5.09†	22.12 ±2.59	15.03 ±1.97	13.80 ± .87	12.75 ±1.28	8.67 ± .60	18.90 ±3.08
Mean survival days	2.6 ± .1	3.4 ± .1	4.3 ± .1	4.7 ± .7	5.1 ± .2	7.1 ± .3	5.3 ± .3
Day of mean death frequency	4.2 ± .1	5.2 ± .2	6.6 ± .3	6.6 ± .3	8.0 ± .5	6.9 ± .4	7.3 ± .3

* Two days after x-irradiation.
† S.E. of mean.
( ) Number of animals.

graded doses of cortisone acetate (Cortone, Merck). The doses used were .005, .05, .5 and 1 mg. per mouse per day. The suspended hormone was made up in constant volumes of 0.2 cc. pyrogen free saline. This total volume was given in two intraperitoneal injections each day until death. Therapy was discontinued on the survivors on the fortieth day after x-irradiation.

The experimental groupings, number of animals in each group and a summary of results are presented in Table 1. The non hormone-treated control groups include the following three categories: x-irradiated adrenalectomized (I), x-irradiated intact (VI), and non-irradiated adrenalectomized (VII) animals. Differences in mortality between the various groups were analyzed by making standard statistical comparisons of the differences in final mortality and in the positions and slopes of the rectilinear segments of the cumulative mortality curves as fitted by the method of least squares.

RESULTS

The cumulative mortality curves of the various groups are depicted in Figure 1. Marked increases in mortality were observed in all those groups which were subjected to both adrenalectomy and x-irradiation. The greatest death rate, however, was observed in that group (I) which did not receive cortisone. Cortisone replacement exerted certain modifications on the mortality of the irradiated-adrenalectomized animals, which led to a progressive rightward displacement of their curves of mortality toward those of the non-irradiated adrenalectomized and intact-irradiated control groups (VII and VI). Significant differences in final mortality at 28 days ($P < .05$) were found only between the intact-irradiated group (VI) and Groups IV, V, VII, but not between the latter three. The modifications of the curves of mortality of the irradiated-adrenalectomized cortisone-treated animals were studied further by comparing the slopes of the rectilinear segments of the mortality curves. The rectilinear portions of the curves as fitted by the method of least squares are shown in Figure 2. The slope values are given in Table 1.

Statistical comparisons of the slopes of the various lines showed signif-

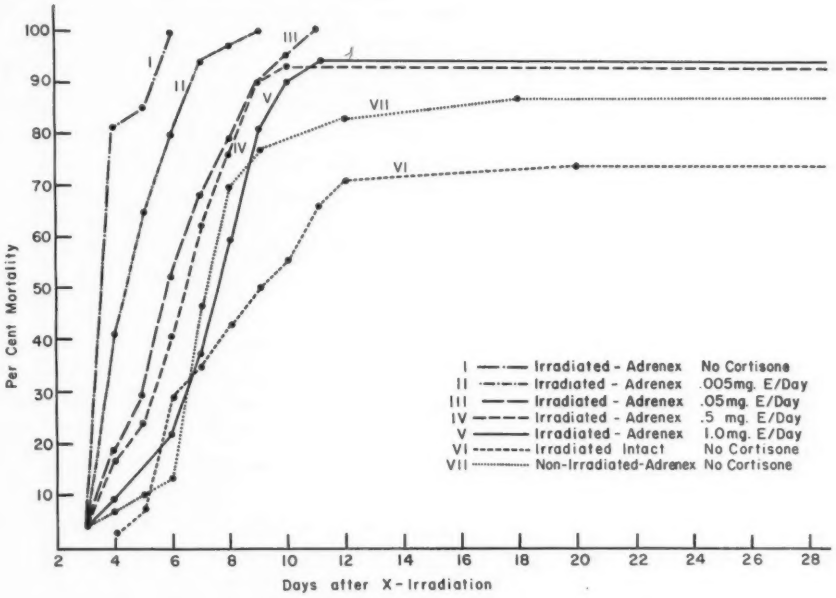
Per Cent Mortality
Days after X-Irradiation
I —·—·— Irradiated - Adrenex No Cortisone
II —·—·— Irradiated - Adrenex .005mg. E/Day
III —— Irradiated - Adrenex .05mg. E/Day
IV – – – Irradiated - Adrenex .5 mg. E/Day
V ——— Irradiated - Adrenex 1.0mg. E/Day
VI ·····Irradiated Intact No Cortisone
VII ····· Non-Irradiated-Adrenex No Cortisone

FIG. 1. Curves of cumulative mortality of intact and adrenalectomized x-irradiated mice, non-treated and treated with graded doses of cortisone.

icant differences between that of the line for Group VI and those of Groups VII and I to V, inclusive. This demonstrates significant differences between the cumulative rates of mortality of the irradiated intact group and the non-irradiated adrenalectomized, irradiated-adrenalectomized, non-treated, and hormone-treated groups.

The slopes of the mortality curves of the experimental Groups I and II (non hormone-treated and treated with .005 mg. hormone/day) were equal to that of the adrenalectomized non-treated Group VII; but differed significantly in position, being far to the left of that of the adrenalectomized control group. This is suggestive of a similarity in the mechanisms leading to death in the three groups, however, the x-irradiated animals succumbed sooner.

The difference between the slopes of any two adjacent lines in the series I to V, inclusive, were not significant. However, as the cortisone dose was increased, there was a progressive decrease in the slopes of the mortality curves which resulted in a highly significant difference between the slopes of lines I and V; also, there was a progressive increase in the latent periods between the time of irradiation and the onset of death, which led to the rightward displacement of the mortality curves.

The relationship between the decrease in the slopes of the cumulative mortality curves and the increase in dose of hormone is depicted in Figure 3, in which slope values are plotted against the log-dose of hormone. The shaded bars represent the mean and standard errors of the mean slopes of the mortality curves of the control groups (VI and VII). The points on the curve represent the slope values as obtained in each of the five irradiated-adrenalectomized groups (I to V). The figure illustrates the significant differences between the slope of the mortality curve of the irradiated-intact control group and those of the adrenalectomized control and the five irradiated-adrenalectomized groups. It also shows that the slopes of the mortality curves of all the irradiated-adrenalectomized groups, despite cortisone treatment, were within the range of that of the adrenalectomized control group. However, there was a significant relationship between the increase in dose of hormone and the decrease in slopes of the mortality curves of the irradiated-adrenalectomized hormone-treated animals. The steepest slope was observed in the non hormone-treated experimental group; .005 mg. cortisone/day reduced the slope of mortality to equal that of the adrenalectomized control group, but the greatest reduction in slope, per unit of hormone used, was obtained with .05 mg. of hormone per day.

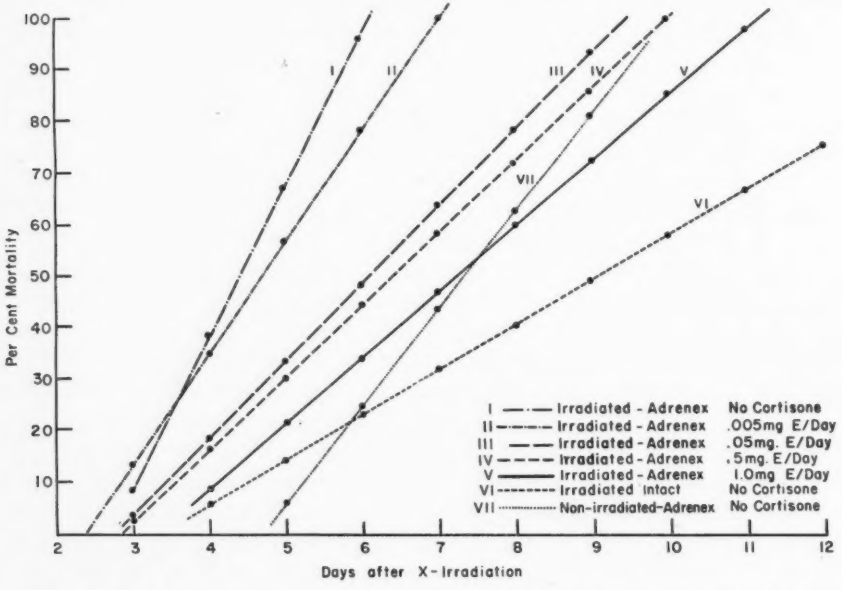

FIG. 2. Fitted rectilinear segments of the curves of cumulative mortality of intact and adrenalectomized x-irradiated mice, non-treated and treated with graded doses of cortisone.

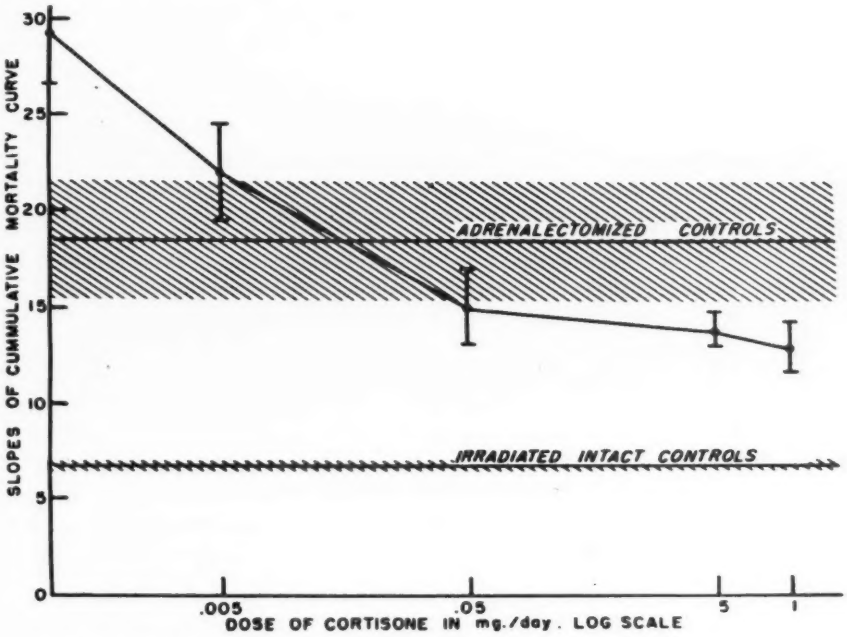

FIG. 3. Log dose-response relationship between the slopes of the cumulative mortality curves and graded doses of cortisone of groups of x-irradiated-adrenalectomized, hormone-treated mice. Shaded bands represent mean and standard errors of means of control groups.

Doses ten and twenty times greater than this did not induce further significant reductions in the slopes of the mortality curves.

The progressive changes in the position and slopes of the mortality curves induced by the graded doses of cortisone resulted in significant direct relationships between the dose of hormone and the delay in death times (DT), increase in mean survival days, and the delay in the day of mean death frequency. These relationships are depicted in Figures 4, 5, and 6.

Comparison of the death times (DT) of 30%, 50%, 70% of the animals in each group (Fig. 4), as determined from the graphs of the fitted lines, shows significant direct rectilinear relationships between the delay in death times and the log-dose of hormone. Similar relationships were found between the log-dose of hormone and the increase in mean survival days and the delay in the days of mean death frequency (Figs. 5 and 6). In each of these figures the differences between the values for the control groups, and also the slopes of the log-dose response lines, are highly significant. Four

irradiated-adrenalectomized animals treated with the highest doses of cortisone survived 40 days, but expired within 7 days after withdrawal of treatment.

DISCUSSION

The results presented here are in agreement with those of Cronkite and Chapman (1950) that adrenal cortical insufficient mice and rats (Edelmann, 1951a) are more sensitive to the effects of x-irradiation, and are in accordance with the many reports that adrenalectomized animals are less resistant to many types of noxious stimuli (reviewed by Perla and Marmorston, 1941; Doughtery, 1951).

The restoration of the resistance of adrenalectomized animals to the effects of thermal injury (Goldthorpe and Dougherty, 1951), bacterial infections, toxins and allergy (Dougherty), has been demonstrated by the administration of cortisone or hydrocortisone. Administration of adrenal cortical hormones to intact animals, however, does not necessarily endow them with an increased resistance to stressful stimuli (Perla and Marmorston 1941; Dougherty, 1951). In order to significantly demonstrate the role of adrenal cortical secretions in influencing the resistance to any stressful

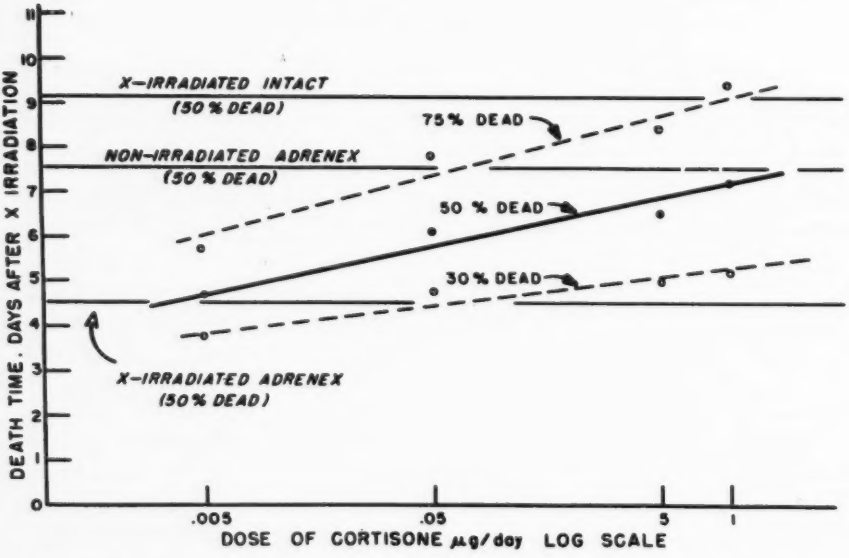

FIG. 4. Log dose-response relationships between the death times (days after x-irradiation) and graded doses of cortisone, at 30, 50 and 70 per cent levels of mortality, in groups of x-irradiated-adrenalectomized, hormone-treated mice. Horizontal lines represent death time of 50 per cent of the control groups.

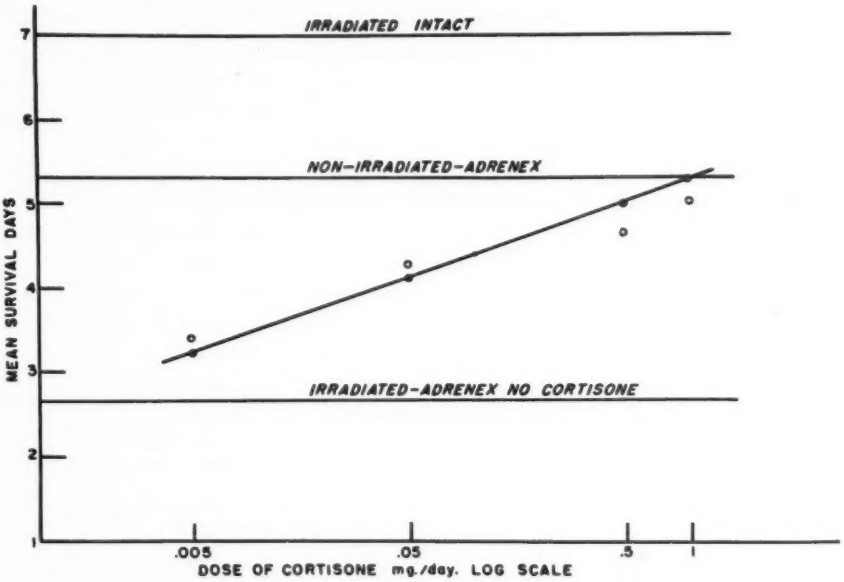

FIG. 5. Log dose-response relationships between the mean survival days and graded doses of cortisone in groups of x-irradiated-adrenalectomized, hormone-treated mice. Horizontal lines represent mean survival days of control groups

stimuli, it must be shown that the resistance of adrenalectomized-hormone-treated animals must be greater or less than that of the adrenalectomized non-treated controls.

Treatment of intact animals with foreign proteins (Hektoen, 1918), estradiol (Treadwell, *et al.*, 1943), adrenal cortical extract (Graham *et al.*, 1950), ten days prior to x-irradiation has been found to decrease mortality. This effect has been attributed to the possible non-specific stimulating effects of these substances, serving to increase the radioresistance of the animals (Graham *et al.*, 1950). Mirand *et al.* (1952), using intact mice, reported a protective influence by cortisone and desoxycorticosterone when either of these compounds was given before or after exposure to 500r head x-irradiation. However, Graham *et al.* (1950) did not find any significant modifications in the survival rates of intact animals repeatedly injected with either aqueous or lipid adrenal cortical extract, 17-dehydrocorticosterone or desoxycorticosterone acetate following total-body exposure to x-irradiation doses ranging from 400r to 600r. It is generally recognized that the organism responds to a wide variety of stressful stimuli by an increased "utilization" of cortical hormone, which is accompanied by rapid and dynamic alterations in the functional activity of the

adrenal cortex (reviewed by Sayers, 1950). Thus, it is difficult to evaluate the influence of exogenous adrenal cortical hormones *per se* in animals subjected to x-irradiation or any stressful stimuli, where, in the experimental procedures the animals are also under the influence of their own intact pituitary-adrenal system.

More valid evaluation of the role of the adrenal cortical hormones in x-irradiation injury may be made using adrenalectomized animals. Adrenalectomy eliminates the animals' own hormone supply, thus excluding the effects of numerous non-specific variables such as diet, season, alterations in temperature, hormone suspending agents, etc. (Santisteban and Dougherty, 1954). In this study, the need for the adrenal cortical secretions during the early period following x-irradiation was shown by the shortened latent period between x-irradiation and the onset of death, and the increased rate of mortality which occurred in those animals which were x-irradiated and adrenalectomized. In intact animals the need for these secretions would be fulfilled by an increased adrenal cortical activity following exposure. This would account for the histochemical and histological changes in the adrenal gland (Englestad and Torgerson, 1939; Dougherty and White,

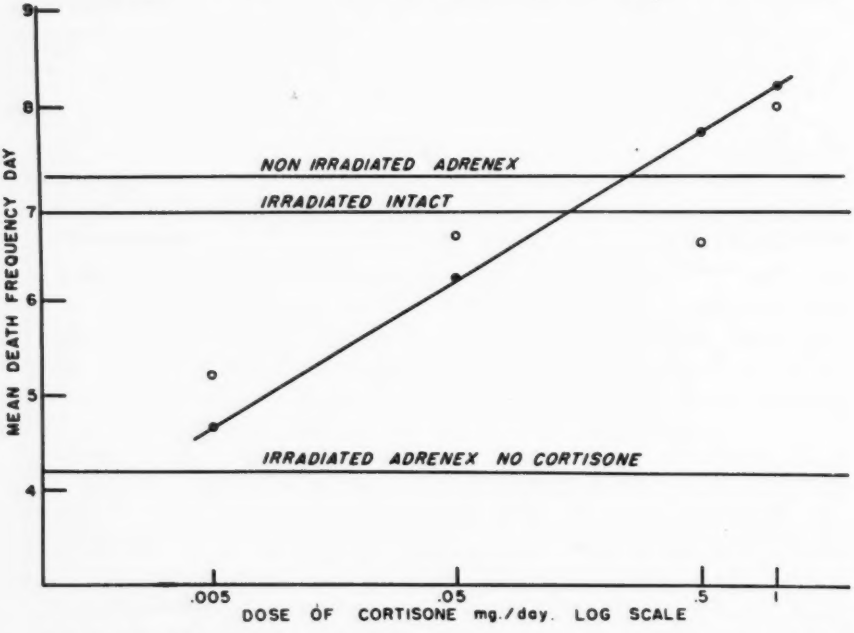

FIG. 6. Log dose-response relationship between mean death frequency days and graded doses of cortisone in groups of x-irradiated-adrenalectomized, hormone-treated mice. Horizontal lines represent mean death frequency days of control groups.

1946; Patt *et al.*, 1947), increased urinary excretion of neutral 17-ketosteroids (Lawrence, 1949) and increased level of 17-hydroxycorticosteroids in the adrenal vein (Bowers *et al.*, 1952) which have been observed following x-irradiation.

The ability of exogenous hormone to partially restore the resistance of the x-irradiated adrenal deficient animals was shown by the progressive decrease in the cumulative rates of mortality and the increase in the latent periods between x-irradiation and the onset of death. These results are in accord with the report that the radioresistance of adrenalectomized rats was increased by use of adrenal cortical extracts and DCA (Edelmann, 1951a), but differ from those of Straube *et al.* (1949), who, using mice which had been adrenalectomized 15 to 17 days prior to x-irradiation, found that the radioresistance of intact and adrenalectomized animals with or without exogenous adrenal cortical steroids were similar. However, studies of the mortality and the growth of lymphatic tissue (as determined by increase in lymphatic organ weight), subsequent to adrenalectomy in CBA mice, indicate that animals which survive over 15 days postoperatively may no longer be adrenal deficient (Santisteban, 1951). Animals which survived over 15 days following adernalectomy often remained alive for an indefinite period. In such animals the amount of lymphatic tissue decreased to the level of that in the intact controls, and the well known atrophy of lymphatic tissue, which accompanies stress in intact animals, was also observed (Santisteban, 1951). The adrenal cortical hormones have been shown to be capable of maintaining adrenalectomized animals and there has been found an excellent correlation between adrenocortical hormone level and lymphatic organ size (Santisteban and Dougherty, 1954); therefore, the survival of adrenalectomized animals, in which the lymphatic tissue response returns to normal, has been attributed to the regeneration of accessory or residual adrenal cortical tissue (Santisteban, 1951). These findings are in agreement with the plateauing of the mortality curve of the non-irradiated, non-treated, adrenalectomized control goup, as observed in this study on the twelfth day after operation (Fig. 1, Group VII).

It is difficult to determine the immediate causes of death in animals subjected simultaneously to adrenalectomy and x-irradiation. The subjective symptoms of death in the experimental animals which were non-treated or treated with the lowest dose of cortisone were more nearly like those observed in the adrenalectomized controls; *e.g.*, anorexia, cachexia, debilitation, but without the rectal hemorrhages characteristically observed in the x-irradiated controls. The experimental animals which were treated with doses of cortisone greater than .005 mg./day appeared healthy and active until about twelve hours preceding death during which time they too devel-

oped symptoms of adrenal cortical insufficiency as well as the hemorrhagic condition.

Further insight into the mechanisms leading to death may be gained indirectly by comparing the slopes and positions of the curves of mortality. Differences in slopes are due to differences in death rates; whereas, those in position are due to differences in death times. These differences may in turn be interpreted as being related to differences in the mechanisms causing death and in the resistance of the animals. Differences in the resistance and conditions leading to death are thus suggested for the intact x-irradiated animals, whose mortality curve differed significantly in slope from those of the other control and experimental groups. It is also suggested that death in the x-irradiated adrenalectomized animals was primarily associated with an adrenal cortical insufficiency; thus the similarities in the slopes of their mortlity curves to that of the adrenalectomized control group. The x-irradiated animals, however, were more sensitive to the lack or insufficiency of the adrenal secretions, as evidenced by their shortened latent periods before the onset of death and the leftward displacement of their mortality curves. Cortisone replacement therapy partially restored the resistance of the experimental animals. This was demonstrated by the reduced slopes and rightward shift of the mortality curves of the hormone-treated experimental groups toward that of the irradiated intact group. The death of the irradiated-adrenalectomized hormone treated survivors upon the withdrawal of cortisone treatment lends further support to the above inference.

The quantitative relations between the observed effects and the dose of administered hormone was demonstrated by (1) the significant inverse relationships between the log dose of hormone and the decrease in slopes of the mortality curves of the experimental groups, and (2) the significant direct rectilinear relationships between the log dose of hormone and the increase in mean survival time, delay in the day of mean death frequency and delay in the death times at any per cent level of mortality.

The importance of using a series of graded doses in studying the effects of pharmacological agents in x-irradiation was pointed out by Ellinger (1952) and is clearly demonstrated in this report. In our laboratory the protective effects of graded doses of cortisone have been evaluated in relation to thermal injuries, bacterial and mycotic infections in adrenalectomized CBA mice (Dougherty). In each case, optimal protection was obtained within the dose range .01 to .05 mg. of hormone per day. Daily doses of .05 mg. of cortisone were also found to maintain the circulating lymphocyte count (Kumagai and Dougherty, 1951) and the thymus weight (Santisteban, 1953) of nonstressed adrenalectomized mice at the level of the intact controls. From this evidence, it has been assumed that .05 mg. cortisone,

given in two daily intraperitoneal injections, effectively fulfill the normal daily requirement of cortisone in the intact, non-stressed mouse (Santisteban and Dougherty, 1954). In this study the optimal protective dose per unit amount of hormone used was also .05 mg./day. Doses ten and twenty times greater did not induce any further increase in resistance, and full restoration of resistance to the level observed in the intact irradiated group was not obtained. This was clearly demonstrated by the significant differences between the slopes of the mortality curves of the experimental animals receiving .5 or 1.0 mg. of hormone/day and the intact x-irradiated animals. The inability of cortisone to fully restore the resistance of the irradiated-adrenalectomized animals is in accord with the report that .2 mg. cortisone/day given for 10 days failed to increase the radioresistance of intact mice (Smith *et al.*, 1950). A low level of cortisone decreases resistance, but a hormone level greater than optimal does not make an animal super resistant.

It has thus been demonstrated that the absence of the adrenal gland enhances the mortality of x-irradiated mice and that limited beneficial effects may be obtained by cortisone replacement therapy. These results suggest that the adrenal cortical secretions are essential in maintaining the resistance of animals subjected to a lethal x-ray dose. Failure of cortisone to restore full resistance, however, implicates the involvement of other factors (disturbance of hormonal balance, other metabolic disturbances, secondary infections, degree of tissue injury, etc.) in x-irradiation injury.

SUMMARY

The influence of cortisone in the acute x-irradiation syndrome was studied by making statistical comparisons of the cumulative mortality curves of groups of x-irradiated (650r whole-body) adrenalectomized CBA mice, some non-treated and others treated with intraperitoneal injections of graded doses of the hormone.

The final mortality and the slope of the cumulative mortality curve of the irradiated-adrenalectomized non hormone-treated group were significantly greater, and the latent period between x-irradiation and onset of death shorter than those of either the hormone-treated experimental groups or the intact irradiated and adrenalectomized, non-irradiated, non-treated controls. Cortisone replacement therapy progressively restored the resistance of the irradiated-adrenalectomized animals; as the hormone dosage was increased there was an increase in the latent periods between x-irradiation and the onset of death, and a progressive decrease in the slopes of the mortality curves. The latter were decreased to the level of the adrenalectomized but not to that of the irradiated intact controls. The greatest reduction in slope of mortality curve, per unit amount of admin-

istered hormone, was obtained with a 0.05 mg./day. Doses ten and twenty times greater failed to induce any further significant reductions. The protective effect of cortisone was further evidenced by significant rectilinear relationships between the log-dose of hormone and the increase in mean survival time, delay in the day of mean frequency, and in death times at any per cent level of mortality.

Analysis of the slopes of the fitted mortality curves and comparisons of the subjective symptoms of death suggest that the increased mortality in the adrenalectomized x-irradiated animals was associated primarily with an adrenal cortical insufficiency and that the events causing death in the irradiated-intact animals differed from those in the x-irradiated-adrenalectomized groups, despite cortisone treatment.

It thus seems that the adrenal cortical secretions are essential for maintaining the resistance to x-irradiation injury, and in the event of a concomitant total adrenal cortical insufficiency, cortisone may partially restore resistance.

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PRODUCTION OF POLYCYTHEMIA IN HYPOPHYSECTOMIZED RATS BY THE PITUITARY ERYTHROPOIETIC FACTOR[1]

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EVIDENCE has recently been presented that the pituitary contains an erythropoietic factor which, administered to hypophysectomized rats, repairs the post-hypophysectomy anemia (Contopoulos, A. N., D. C. Van Dyke, M. E. Simpson, J. F. Garcia, R. L. Huff, B. S. Williams and H. M. Evans, 1953; Contopoulos, A. N., D. C. Van Dyke, S. Ellis, M. E. Simpson, J. H. Lawrence, and H. M. Evans, 1954; Contopoulos, A. N., M. E. Simpson, D. C. Van Dyke, S. Ellis, J. H. Lawrence and H. M. Evans, 1954; Simpson, M. E., D. C. Van Dyke and B. S. Williams, 1954; Van Dyke, D. C., A. N. Contopoulos, B. S. Williams, M. E. Simpson, J. H. Lawrence and H. M. Evans, 1954). This pituitary erythropoietic factor does not appear to act through any of the known pituitary target organs. None of the known pituitary hormones or the hormones of its target organs have been shown to cause polycythemia when injected into hypophysectomized rats (Van Dyke, D. C., A. N. Contopoulos, B. S. Williams, M. E. Simpson, J. H. Lawrence and H. M. Evans, 1954). Furthermore, none of the known erythropoietic stimulants (anoxia or cobalt) were shown to produce polycythemia in the hypophysectomized rat. As it has been shown that injection of the pituitary erythropoietic factor in normal animals induces a red cell volume in excess of normal, it was important to determine whether its injection into hypophysectomized rats would result in polycythemia. It is the object of this report to show that polycythemia does occur in hypophysectomized rats after injection of the pituitary erythropoietic factor.

MATERIALS AND METHODS

Female rats of the Long-Evans strain were hypophysectomized at 26–28 days of age and kept for forty-five days for the development of the characteristic post-hypophysectomy anemia. Afterwards, they were injected subcutaneously daily for 14 days with 100 μg. of the pituitary erthropoietic factor suspended in beeswax. Hypophysectomized con-

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TABLE 1. PRODUCTION OF POLYCYTHEMIA IN HYPOPHYSECTOMIZED RATS BY THE PITUITARY ERYTHROPOIETIC FACTOR

Group	No. of rats	Body weight	Hemoglobin	Hemoglobin/100 gm. body weight	Hematocrit	Total red cell volume	Red cell volume/100 gm. body weight
		gm.	gm./100 ml	gm.	%	ml	ml
Hypophysectomized Controls	7	84±2	10.3±0.3	0.50±0.01	27.7±1.2	1.14±0.10	1.36±0.08
Hypophysectomized injected with Erythropoietic Factor	6	87±2.5*	12.3±0.3	0.61±0.01	57.3±3.5	2.52±0.17	2.90±0.12
Normal Controls	7	205±6	12.9±0.3	0.65±0.01	45.5±1.3	4.80±0.15	2.34±0.05

* Standard Error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

trols were injected with the beeswax vehicle or with saline, and since no difference in their hematological values was found the results are combined in Table 1. Normal rats were kept as controls. The preparation containing the pituitary erythropoietic factor was obtained from sheep pituitaries (Simpson, M. E., D. C. Van Dyke and B. S. Williams, 1954).

At the end of the experimental period, red cell volume determinations were done using the Fe^{59} labeled red cell dilution method.[2]

The completeness of hypophysectomy was determined by examination at autopsy of the pituitary site and by histological examination of the target organs.

All rats were kept on a standard laboratory diet given ad libitum,[3] and were kept in a constant temperature room throughout the experiment (75 ±2° F.).

RESULTS

Table 1 shows that hypophysectomized rats injected with the pituitary erythropoietic factor showed a marked increase in red cell volume, hemoglobin and hematocrit. These values were not only brought back to normal but exceeded normal value. The hematocrit of 57.3% and the red cell

[2] The labeled red cells were obtained from a donor of the same strain previously injected with Fe^{59}. The experimental animals were injected through the saphenous vein with 0.1 ml. of donor blood which contained 0.03 microcuries Fe^{59} in the erythrocytes. After allowing six minutes for the blood to mix, the abdomen was opened and a sample of blood was drawn from the vena cava into a heparinized syringe. A known volume of this blood was pipetted into a vial and its activity counted directly in a scintillation counter (Anger, H. O., 1951). The total blood volume was calculated from the fraction of the injected activity found in the sample of known volume. The total blood volume thus calculated, multiplied by the hematocrit (determined in Wintrobe tubes), gave the total circulating red cell volume. The values for the total circulating red cell volume were then divided by the values for the body weights of the animals, and the results are presented in terms of ml. of red blood cells per 100 gm. body weight. The hemoglobin concentration was determined by the method of Turner (1946).

[3] The standard laboratory diet (diet XIV), given dry, was supplemented by a wet mash of diet I given daily. Lettuce was given 2 to 3 times weekly. Diet XIV consists of 68.5% wheat, 5% casein, 10% fish meal, 10% alfalfa leaf meal, 5% fish oil, 1.5% NaCl, KI added (analysis 1 μg. iodine per g. diet). Diet I consists of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.0% fish oil, 0.75% NaCl, 1.5% $CaCo_3$, KI added (analysis 1 μg. iodine per g. diet).

volume of 2.90 ml./100 gm. of body weight were above the corresponding values in the normal controls. The red cell volume was approximately 25% higher than the normal value and 115% higher than the value in hypophysectomized controls. The hematocrit was also 25% above the corresponding normal controls and 100% above the value in hypophysectomized controls.

The peripheral blood of injected hypophysectomized rats also showed the stimulation of red cell production by the increased reticulocyte count, and by the presence of circulating immature red cells. The bone marrow in the femur was vascular and cellular in contrast to the pale fatty marrow of controls. The detailed histology of the bone marrow, spleen, liver and blood will be reported elsewhere (Rapatz, E. R., A. N. Contopoulos, S. Ellis, M. E. Simpson and H. M. Evans, 1954).

DISCUSSION

Of all the known pituitary trophic hormones only adrenocorticotrophically active preparations were able, when injected in large doses into hypophysectomized animals, to repair the post-hypophysectomy anemia (Garcia, J. F., D. C. Van Dyke, R. L. Huff, P. J. Elmlinger and T. M. Oda, 1951). Though high doses were employed, no polycythemia was produced in the hypophysectomized animals even after injections for 34 to 116 days (Garcia, J. F., D. C. Van Dyke, R. L. Huff, P. J. Elmlinger and T. M. Oda, 1951). Recently (Contopoulos, A. N., D. C. Van Dyke, S. Ellis, M. E. Simpson, J. H. Lawrence and H. M. Evans, 1954), evidence has been presented supporting the concept that adrenocorticotrophic hormone acting through the adrenals is not the principle stimulating erythropoiesis, but that a contaminant in these preparations is responsible for the increased red cell production.

The combination of the pituitary target organ hormones and high protein diet partially repaired the anemia in hypophysectomized rats but in no case brought the hematological values above normal (Crafts, R. C., 1949).[4]

The injection of cobalt sulfate into hypophysectomized rats brought the blood values to the normal level after 53 days (Garcia, J. F., D. C. Van Dyke, and N. I. Berlin, 1952). There was a 58% increase in red cell volume per 100 gm. body weight. Crafts (1949), reported that daily injections of 100 μg. of cobalt nitrate did not prevent the development of post-hypophy-

[4] Hypophysectomized rats (2 months post-operative) injected daily for 14 days with 1.0 mg. of testosterone propionate and 5 μg. of thyroxine, and kept on 64% protein diet, showed only a 42% increase in red cell volume and hematocrit. This increase did not restore the hematological values to the normal level (Contopoulos, Simpson, Lawrence, Evans, Unpublished Data).

sectomy anemia, as judged by erythrocyte counts, hemoglobin and hematocrit. At higher doses the development of anemia could be prevented, and the animals even showed an increased red cell count, hemoglobin and hematocrit. However, these doses were sufficiently toxic to the hypophysectomized animals to cause a loss in body weight; therefore any hematological determinations based on peripheral concentration methods may be subject to error because of hemoconcentration. In Crafts' own judgment, "the higher erythrocyte count in the hypophysectomized rats than in the normals with cobalt therapy is, therefore probably an indication of the hemoconcentration in the hypophysectomized animal."[5]

The other stimulus known to affect erythropoiesis in the rat is hypoxia. When hypophysectomized rats were put in the chamber at 22,000 feet, daily for 14 days, 6 hours a day, they showed a 40% increase in circulating red cell volume over that of the hypophysectomized controls maintained at s level (Van Dyke, D. C., A. N. Contopoulos, B. S. Williams, M. E. Si pson, J. H. Lawrence and H. M. Evans, 1954). When they were exposed to the same altitude for 22 days, a 57% increase occurred so that the cell volume was brought back to normal level.

Of all the factors reported to be stimulants of erythropoiesis in the hypophysectomized rat, none has been found to induce polycythemia. The present demonstration that the pituitary erythropoietic factor injected in small doses for short periods of time produces polycythemia lends further support to the concept of the importance of this substance in erythropoiesis. Its presence is necessary as a stimulant in red cell production and its absence interferes with the normal output of red cells by the bone marrow. Its role in the development of the physiological neonatal anemia has been reported elsewhere (Contopoulos, A. N., D. C. Van Dyke, S. Ellis, M. E. Simpson, J. H. Lawrence and H. M. Evans, 1954; Contopoulos, A. N., and J. H. Lawrence, 1954).

SUMMARY

Injection of the pituitary erythropoietic factor in hypophysectomized rats resulted in the development of polycythemia. The rats were females of the Long-Evans strain, hypophysectomized when 26–28 days of age and maintained for at least a 45 day postoperative period in order to allow for development of the characteristic post-hypophysectomy anemia. Injection of a daily dose of 0.100 mg. of the erythropoietic factor for 14 days not

[5] Cobalt injected for 14 days at the doses reported in the literature as stimulating erythropoiesis (Crafts, 1952; Garcia, Van Dyke and Bezlin, 1951) produced only a 10% increase in red cell volume, hematocrit and hemoglobin (Contopoulos, Simpson, Lawrence, Evans, Unpublished Data).

only repaired the anemia, but resulted in hematological values (hemoglobin, hematocrit and total red cell volume) exceeding those of the normal controls. Since none of the known pituitary trophic hormones, even when given at high levels over a long period, has been able to achieve this result, the production of polycythemia in hypophysectomized rats has been interpreted as giving additional support to the concept that the pituitary erythropoietic factor is a substance distinct from all other hypophyseal hormones.

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A COMPARATIVE STUDY OF ALDOSTERONE AND OTHER ADRENAL STEROIDS IN ADRENALECTOMIZED DOGS[1]

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THE effectiveness of therapeutic doses of cortisone is considerably increased for certain types of disability by administering the free alcohol of the steroid in a weak aqueous-alcohol solution (10%), instead of using the more slowly absorbed microcrystalline suspension (Swingle, Fedor *et al.*, 1953). The unequivocal superiority of this solubilized cortisone over the suspension in preventing the otherwise fatal anaphylactoid response of 24-hour adrenalectomized rats to infusions of the polysaccharide dextran, led us to undertake a comparative study of the efficacy of similarly solubilized aldosterone,[2] DOC, cortisone and hydrocortisone, when tested on adrenalectomized dogs.

Previous studies (Swingle, Barlow *et al.*, 1953) had shown that dogs deprived of their adrenals require large doses of commercial cortisone suspension (Merck's Cortone) for maintenance. Thus, it was demonstrated that daily intramuscular injections of 0.93 mg./Kg./day are inadequate to retain a normal serum electrolyte pattern, although twice this amount, or 1.86 mg./Kg./day is sufficient to do so. A daily dose of 3.72 mg./Kg. was ample to restore to normal any distortions of the electrolyte levels. In our experience, therefore, 1.86 mg./Kg. of the suspension given once daily proved to be an adequate maintenance dose for the dogs. To anticipate one result obtained in the present experiments, it can be stated that such a daily dose of microcrystalline cortisone is approximately 6 times more than is required for maintenance when the solubilized material is used.

The adrenal steroids employed in these experiments are well known and, with the exception of aldosterone, need no further comment. This potent mineralocorticoid has only recently been isolated from cortical extracts in

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[2] We are indebted to Professor T. Reichstein of the University of Basle, Switzerland, for the crystalline aldosterone. Ciba Pharmaceutical Products, Inc., supplied the DOC and DCA and Schering Corporation the hydrocortisone.

pure crystalline form (Simpson *et al.*, 1953; Simpson and Tait, 1953; Mattox *et al.*, 1953) but at present is not readily available for physiological or chemical study owing to scarcity of material. We greatly appreciate Professor Reichstein's generosity in sending us sufficient crystals for testing 4 adrenalectomized dogs.

Chemical degradation of the crystalline product by Simpson *et al.* (1954) showed the compound to be 11β,21-dihydroxy-3,20-diketo-4-pregnene-18-al, and that it reacts mainly as the 11-hemiacetal when in solution.

METHODS

A total of 16 dogs was used; 4 animals were assigned for testing each of the various steroids. They had been without adrenals for periods ranging from 1–4 years previous to use in these experiments. During this interval they were utilized numerous times for tests involving cycles of insufficiency and recovery. In the interim between experiments the dogs were maintained in normal health and vigor by a single, daily, intramuscular injection of 0.5 mg. DCA in oil.

Since the dietary regimen is an important element in investigations concerned with the comparative potency of corticoids, the total quantities of Na and K fed in the daily ration were determined at intervals and maintained at 1.47 gm. of Na and 0.94 gm. of K. The ingredients of the diet and amounts fed each day were essentially the same as those reported in an earlier communication (Swingle *et al.*, 1952), except that fresh ground beef was substituted for the horse meat given in the original diet.

The test dogs, with the exception of 2 aldosterone-treated animals, were kept at each dose level for a minimum of 10 days, after which the arterial pressure was determined, blood samples taken for analysis and the daily dosage reduced by 50%. This procedure was followed until eventually the quantity of steroid was reduced to a level inadequate for maintenance and insufficiency symptoms appeared.

The aldosterone and DOC crystals were each dissolved in warm 95% ethanol which was then diluted dropwise with distilled water to yield 30% solutions in which the concentration of aldosterone was 25 μg./cc. and the DOC was 1.5 mg./cc. These 30% alcohol solutions were kept as stock and the daily dosage was then removed, diluted to 10% and given subcutaneously in divided doses, one half at 9:30 A.M. and the remainder at 4:30 P.M.

Ten per cent alcoholic solutions of cortisone and hydrocortisone containing 2.5 mg./cc. were prepared daily by the method outlined above and kept warm to prevent precipitation. These steroids were administered in similar manner to aldosterone and DOC.

Adequate reference to the methods employed for determination of the arterial pressure and various blood constituents, have been made in earlier publications and will not be repeated here (Swingle *et al.*, 1952a). Serum Na and K were measured by means of the Perkin-Elmer flame photometer (Model 52C). The dogs were all table trained for blood pressure studies.

RESULTS

Aldosterone. Four dogs were used for testing this steroid; 2 were given an initial dose of 25 μg./dog/day for 10 days (nos. 1 and 3); the remaining 2 (nos. 2 and 4) started at 10 μg./day. Study of one animal (no. 4), was

terminated at the end of 7 days due to lack of material. The essential data are recorded in Table 1.

The minimum maintenance dose which sufficed to keep the serum electrolytes, blood urea nitrogen and arterial pressure normal, varied between 12.5 and 6.2 μg./dog/day. One animal (no. 3) remained free from any alteration in blood consitutents and blood pressure at a dose level of 6.2 μg.; another (no. 2), was adequately maintained at this same dosage except for a slight fall in blood pressure to 90 mm. Hg; a third (no. 1) required 12.5 μg./day, and a fourth (no. 4), remained normal throughout 7 days at 10 μg./day.

The onset of adrenal insufficiency is a process of gradual deterioration in these dogs and the early manifestations varied only slightly from animal

TABLE 1. EFFICACY OF AQUEOUS-ALCOHOL SOLUTIONS OF ALDOSTERONE AND DOC FOR MAINTAINING ADRENALECTOMIZED DOGS

Dog No.	Dose, μg./day	Injection period, days	Body weight, kg.	Blood pressure, mm. Hg.	Blood urea N., mg. %	Hb, gm. %	Hmct., %	RBC ×10⁶/ cu. mm.	Blood glucose, mg. %	Serum Na, m.eq./l	Serum Cl, m.eq./l	Serum K, m.eq./l
						Aldosterone						
1	initial		13.84	115	21.7	12.05	29.5	5.72	83.0	142.5	115.5	5.03
	25.0	10	13.61	104	29.0	11.00	31.3	6.08	81.0	144.5	113.5	5.28
	12.5	10	13.49	94	28.2	12.40	36.0	7.24	81.5	141.5	108.3	5.79
	6.2	10	13.61	85	25.6	12.28	31.8	6.06	82.5	139.0	104.6	6.50
	3.1	10	14.06	72	25.3	11.59	30.3	5.85	82.0	—	105.2	6.42
	1.5	3	14.06	59	44.0	12.51	26.9	6.02	91.0*	138.0	109.7	8.70
2	initial		15.56	120	20.9	11.71	29.3	6.19	88.0	141.5	114.9	4.22
	10.0	7	15.56	94	20.8	12.91	31.8	6.52	80.0	143.0	107.3	5.12
	6.2	10	15.31	90	32.3	13.61	34.6	6.21	84.5	140.5	111.3	5.62
	3.1	10	15.31	91	35.1	14.20	36.9	6.63	83.0	135.5	107.3	6.50
	1.5	8	14.85	58	105.0	15.43	36.9	7.03	83.0	136.5	107.2	9.16
3	initial		20.23	100	21.9	10.90	29.9	5.97	90.5	147.0	114.1	4.97
	25.0	10	20.00	101	31.5	10.81	31.9	6.10	83.5	145.5	112.5	5.36
	12.5	10	19.54	104	30.6	12.28	36.7	6.63	82.0	142.0	108.7	5.37
	6.2	10	19.31	103	25.8	11.94	31.1	6.85	81.0	141.0	105.2	5.36
	3.1	10	18.86	82	35.3	12.91	36.9	7.57	86.5	138.0	108.0	8.45
	1.5	2	18.72	64	43.5	14.90	33.8	6.72	76.5	140.0	108.7	8.61
4	initial		16.33	119	22.4	11.29	33.0	5.04	79.5	144.5	116.2	4.64
	10.0	7	15.99	119	26.9	11.82	30.8	5.71	78.5	144.0	110.0	4.95
						Desoxycorticosterone						
5	initial		16.68	117	22.3	10.62	32.8	5.78	83.0	146.0	114.7	4.71
	1000	10	16.56	107	27.1	13.33	35.6	5.61	81.5	149.0	115.5	4.81
	500	10	16.33	98	24.6	13.89	37.3	5.74	81.5	143.5	109.4	4.87
	250	10	16.33	92	25.3	15.43	37.0	6.29	81.5	140.5	107.0	5.77
	125	10	16.11	85	24.5	13.19	33.5	6.64	84.0	134.5	102.0	5.86
	62	10	16.11	68	53.8	13.33	33.3	7.07	82.5	133.0	107.3	7.63
6	initial		16.11	120	15.2	11.29	31.0	5.84	84.0	145.5	115.0	4.79
	500	10	16.22	120	18.1	11.38	29.4	5.54	76.0	144.0	116.0	5.09
	250	10	15.99	119	20.9	11.48	29.3	5.36	80.0	140.0	114.1	5.62
	125	5	15.88	60	22.0	11.71	30.0	5.86	73.0	138.0	108.9	5.13
7	initial		15.42	118	26.7	11.10	29.2	6.71	75.0	145.0	115.5	4.54
	250	10	15.31	112	28.2	11.19	28.7	6.33	76.5	139.5	109.4	4.87
	125	10	15.08	108	30.5	12.63	33.4	7.10	77.5	136.5	109.0	6.05
	62	10	14.74	72	25.8	12.77	32.2	6.45	86.0	130.5	108.0	6.72
	31	10	14.51	66	42.0	12.91	31.4	5.69	91.0	133.5	108.5	8.10
8	initial		19.63	114	21.3	10.90	26.5	5.83	82.0	143.3	112.9	4.24
	250	10	19.88	103	28.8	11.71	29.0	6.15	85.5	140.5	108.7	4.93
	125	10	19.31	112	25.5	10.71	30.9	6.19	83.0	139.5	108.0	5.52
	62	10	19.09	96	30.5	13.75	35.9	6.28	85.5	134.0	109.0	5.79
	31	10	18.86	79	53.2	13.19	33.1	5.75	87.0	136.5	108.0	7.86

Doses italicized are minimum maintenance levels.
* Not a fasting blood sugar.

to animal. In two (nos. 1 and 2), the first hint of subminimum dosage was a decline in arterial pressure. This occurred at a time when the serum Na and K remained well within normal limits. On the other hand, one dog (no. 3), exhibited a marked rise in serum K concomitant with the fall in blood presure. The blood urea nitrogen was maintained normal or at moderately elevated levels until a few days before crisis at which time it rose sharply.

Data obtained from study of the serum K changes revealed that aldosterone is considerably less efficient in preventing accumulation of excess serum K in the blood than it is in retaining Na. Examination of the table shows that of the animals listed, 3 exhibited serum K levels exceeding 8.6 m.eq./l. at a time when the serum Na was either normal (Dogs 1 and 3) or but slightly reduced below the normal range for this cation (Dog 2).

Thus it seems that aldosterone when gradually reduced to subminimum dose levels (3.1–1.5 μg./dog/day) manifests only a weak regulatory action upon those mechanisms concerned with maintaining a normal serum K. This ion accumulates in the blood of the aldosterone-treated dog when the dosage of this steroid is sufficient to maintain normal serum Na levels. Two animals (nos. 2 and 3), developed severe cardiac symptoms and prostration due to hyperpotassemia, although at the time, the Na was normal in one case (140 m.eq./l.) and but slightly reduced in another case (136.5 m.eq./l.).

It seems evident from these data, that adrenal insufficiency and circulatory failure can occur in aldosterone-treated dogs acompanied by severe symptoms of hyperpotassemia (nos. 1, 2 and 3 Table 1), but whithout significant decline in serum Na levels. The fall in Na may be insignificant at a time when other blood constituents have undergone drastic changes, hence serum levels of this cation should not be relied upon to serve as the sole criterion of what constitutes a maintenance dose of this mineralocorticoid. Other factors such as arterial pressure, blood urea nitrogen and clinical condition of the animal must also be taken into consideration.

Desoxycorticosterone. The starting dose of DOC in 10% alcohol ranged from 1000–250 μg./dog/day and was lowered by 50% every 10 days. The minimum daily requirement for this steroid was not the same for the four animals; the maintenance dose for 2 (Dogs 5 and 6) was 250 μg./day, whereas the other 2 (dogs 7 and 8) maintained equally well at 125 μg./dog/day. In earlier tests (Swingle, Perlmutt *et al.*, 1952b), assays of DCA in oily solution were made and the amount found necessary for maintenance varied between 300–500 μg./day when given as a single daily intramuscular injection. However, 2 animals of a group of 7 were kept free from symptoms and showed no deviations from normal in blood pressure or blood constituents at a dose level of 125 μg./day. Hence it is possible to occasionally maintain dogs at these surprisingly low doses of DCA in oil, but the reduc-

tion in the amount of steroid given per day must be very gradual and extend over considerable time.

Animals (Dogs 5, 7, 8) subjected to periodic reductions in dosage of the 10% alcohol solutions of DOC only gradually lose their vigor and appetite even though the arterial pressure may be 70–60 mm. Hg., and the serum electrolyte pattern is distinctly abnormal. They eventually develop severe insufficiency requiring drastic remedial measures but manage to survive a long time at definitely subminimum dosage. It seems probable that the efficiency of the solubilized steroid is due in part to (1) rapid and complete absorption; (2) increased effectiveness when given in divided doses morning and evening; and perhaps (3) adjustment of the dog to gradual reduction in dosage over an interval of several weeks.

Dog 6, Table 1, is an example of vasomotor failure, with resulting circulatory collapse, in an animal maintaining normal serum electrolytes, blood urea nitrogen and hemoconcentration. Loeb (1938), has reported similar findings in Addisonian patients. The writers (Swingle *et al.*, 1954) testing the effects of a potent Na-retaining substance derived from the amorphous fraction and given to us for testing by Dr. E. C. Kendall, occasionally have observed failure of the circulation in dogs presenting normal serum electrolyte patterns.

Cortisone and Hydrocortisone. These two steroids are discussed together since the results of the tests revealed only insignificant differences in the reaction of the dogs to them (Table 2). The daily maintenance dose of both was approximately 5 mg./dog/day. One animal (no. 11) receiving this amount of hydrocortisone daily, displayed low Na and Cl at the termination of the initial 10 day interval, hence 5 mg. was a subminimum dose for this particular dog.

Relatively large amounts of the microcrystalline suspension of cortisone are necessary to keep the serum electrolytes normal; a daily dose of 1.86 mg./Kg./day was found to be necessary (Swingle, Barlow *et al.*, 1953). However when the 10% alcohol solutions of cortisone and hydrocortisone were tested, only a fraction of this amount sustained the animal in normal health and vigor. For instance, the average weight of the 4 dogs listed under cortisone in Table 2 was 16.6 Kg.; if the dosage is calculated on a weight basis, then only 0.3 mg./Kg./day of the aqueous-alcohol solution are required for maintenance. Thus, approximately 6 times more of the cortisone acetate suspension is needed for maintenance than is necessary when the solubilized material is used.

The dogs receiving less than 5 mg. of cortisone or hydrocortisone a day all exhibited marked decline in serum Na and Cl and increases in K. These serum electrolyte alterations are striking (notably the Na and Cl) when compared with the relatively minor modifications occurring in the aldos-

TABLE 2. MAINTENANCE OF ADRENALECTOMIZED DOGS WITH AQUEOUS-ALCOHOL SOLUTIONS OF C_{11}-OXYSTEROIDS

Dog No.	Dose, μg./day	Injection period, days	Body weight, kg.	Blood pressure, mm. Hg.	Blood urea N., mg. %	Hb, gm. %	Hmct., %	RBC ×10⁶/cu. mm.	Blood glucose, mg. %	Serum Na, m.eq./l	Serum Cl, m.eq./l	Serum K, m.eq./l
						Hydrocortisone						
9	initial		16.33	115	21.8	10.52	30.7	5.07	89.5	144.0	120.0	4.64
	5000	10	15.65	110	25.9	13.61	31.9	5.20	79.8	143.3	116.0	4.83
	2500	10	15.42	100	37.0	18.04	40.8	6.49	80.0	137.0	109.0	6.23
	1250	6	15.31	72	49.4	16.46	39.3	6.01	81.5	130.7	107.4	8.61
10	initial	10	14.97	98	16.4	10.20	28.6	5.25	75.5	147.5	113.5	3.47
	5000	10	14.29	87	20.8	10.90	31.7	5.61	79.0	137.0	108.0	5.26
	2500	10	14.06	85	24.7	14.90	37.7	6.41	83.0	131.0	101.5	6.66
	1250	9	13.61	62	42.0	16.01	37.8	6.75	89.5	133.0	100.0	6.83
11	initial		20.34	115	18.1	9.95	33.3	6.42	80.5	142.5	114.0	4.06
	5000	10	19.63	115	23.3	12.63	38.1	7.16	85.5	133.5	102.3	6.25
	2500	10	19.88	91	44.0	13.19	40.0	7.72	88.5	128.7	102.5	8.77
12	initial		16.11	97	18.5	11.00	28.0	5.12	79.5	146.0	116.0	4.94
	5000	10	15.42	98	31.8	15.25	34.5	6.08	80.5	140.8	102.5	5.91
	2500	7	15.31	70	41.0	16.01	37.5	6.86	93.0*	132.0	100.0	8.13
						Cortisone						
13	initial		14.06	120	15.1	10.90	26.6	5.70	75.0	142.0	116.0	3.96
	5000	10	13.26	105	21.4	14.03	42.6	7.40	80.5	136.5	107.3	6.01
	2500	10	13.15	91	22.3	14.55	40.9	6.79	86.5	136.0	100.0	6.99
	1250	7	13.26	67	43.0	—	41.0	6.95	88.5	134.0	105.7	6.83
14	initial		16.44	116	23.6	11.82	32.8	5.59	88.0	146.5	114.6	4.64
	5000	10	15.31	118	29.8	14.55	42.0	6.53	85.5	137.5	100.0	6.50
	2500	10	14.97	79	75.2	15.08	40.0	7.49	83.5	133.5	101.5	7.21
	1250	4	14.97	65	97.2	15.08	36.6	7.06	88.0	132.5	100.4	8.71
15	initial		20.68	109	22.3	12.91	37.0	6.36	85.5	143.0	113.1	4.87
	5000	10	19.43	110	25.0	15.43	37.2	6.86	88.5	139.5	100.0	6.58
	2500	7	19.43	96	42.0	15.78	41.8	8.18	81.0	134.5	99.4	9.32
16	initial		15.20	98	22.7	9.79	28.6	5.25	77.5	143.3	116.5	4.33
	5000	10	13.84	87	26.0	12.63	31.2	5.81	77.5	140.8	105.7	6.01
	2500	7	14.06	86	33.0	15.20	35.4	6.02	85.0	132.5	101.9	7.21

Doses italicized are minimum maintenance levels.
* Not a fasting blood sugar.

terone series given adequate therapy or even doses well below the level which will maintain normalcy.

DISCUSSION

Few investigators have had access to sufficient aldosterone for physiological experimentation, hence the literature dealing with this crystalline steroid is not voluminous. Desaulles *et al.* (1953), were the first to test pure aldosterone and to state that it was 25 times more active than desoxycorticosterone in inhibiting Na excretion in rats. Mattox *et al.*, (1953), reported a similar degree of activity on Na-retention for their crystalline preparation. Other workers (Speirs *et al.*, 1954) also using rats, and employing the Simpson-Tait test, report a potency for aldosterone 120 times that of desoxycorticosterone.

Gross (1954), and Gross and Gysel (1954) used adrenalectomized dogs and compared the potency of the new steroid with that of DCA. They concluded that, "In the adrenalectomized dog, the daily dose of electrocortin (aldosterone) required for maintenance of life is 1.5 to 2.0 μg./Kg. by sub-

cuteneous injection in oily solution." They found aldosterone to be 25-30 times more active than DCA.

In the present study, the average minimum maintenance dose of this compound was approximately 10 μg./dog/day when administered in 10% alcohol in divided doses twice daily (the daily dose required for complete maintenance varies from 6.2 to 12.5 μg./day). Since the least amount of DOC which was necessary to maintain the dogs was 125–250 μg./day it is obvious that aldosterone is approximately 12–25 times more potent than DOC when tested under the conditions of these experiments.

Altering the conduct of the experiments, even in minor respects will undoubtedly give different figures for potency. Thus, Gross and Gysel (1954) obtained data indicating a higher degree of activity of aldosterone as compared with DCA than the potency figures cited in these experiments. However, the conditions under which the two sets of data were obtained are different (1) The Swiss investigators administered aldosterone in oil; (2) the longest period of observation at the minimum dosage was 10 days; (3) the DCA was in oily solution and the maintenance dose varied from 0.7–1 mg. injected subcutaneously once daily, and finally (4) 0.5 gm. of salt was added to the basal ration. If these differences in diet, vehicle for solubilizing the steroid, duration of the test etc., are given due consideration it becomes evident that the data of Gross and Gysel and those presented in Table 1 are not greatly different.

Earlier work done in this laboratory (Swingle, Perlmutt *et al.*, 1952) had shown that the minimum maintenance dose of DCA in oil that will preserve normal arterial pressure, serum electrolytes and hemoconcentration in tests on adrenalectomized dogs which varied in no way from those presented here, was 300–500 μg./dog/day. This is 30–50 times more steroid than the 10 μg. daily requirement for aldosterone.

A larger dose of this mineralocorticoid is required for prevention of the slow decline in arterial pressure (generally the first indication of inadequate dosage) than is necessary to preserve a normal serum electrolyte pattern. It is not an uncommon observation for an animal to show severe symptoms of adrenal insufficiency with low arterial pressure and yet maintain normal serum Na levels.

The most pressing question concerning the newly isolated steroid is whether it differs qualitatively as well as quantitatively from DOC. Too meager a supply of aldosterone is now available and too few experiments have been performed to permit a definitive answer. However, on the basis of what is now known, certain facts appear to be established and suggest that aldosterone differs in several respects from desoxycorticosterone. (1) It is much more potent in retaining Na; (2) According to Dessaules *et al.*,

(1953) it is active in stimulating K excretion, but much less so than DCA; (3) It has been reported (Schuler *et al.*, 1954) that aldosterone is 30 times more active than DOC in increasing the liver glycogen of mice. Gaunt and Associates (1954) state that it shows potency as great or even greater than cortisone when used on rats in the cold stress test. However, according to these authors, it has approximately one-half the activity of the C_{11}-oxysteroids in the eosinophil depletion test.

All of these reports suggest that aldosterone although exhibiting some of the activity characteristic of DOC, nevertheless, apparently posseses a number of other physiological properties which differ from those of desoxycorticosterone.

The minimum maintenance dose of the aqueous-alcohol solutions of cortisone and hydrocortisone was 5000 μg./dog/day. This is 500 times more than the maintenance requirement for aldosterone but is 6 times less than the minimum daily maintenance dose of the microcrystalline suspension of cortisone.

SUMMARY

The minimum maintenance dose of aldosterone and the free alcohols of DOC, cortisone and hydrocortisone, when administered to adrenalectomized dogs in 10% alcohol in divided doses twice daily is as follows: Aldosterone, approximately 10 μg./dog/day; DOC 250–125 μg./dog/day; cortisone and hydrocortisone, 5000 μg./dog/day. Thus, the daily requirement for aldosterone, under the conditions of these experiments, is 12–25 times less than that required for DOC and is but 1/500th of the daily dose necessary for maintenance when either of the C_{11}-oxysteroids are used. If the dogs are assayed using DCA in oil, the minimum dose preserving normality in tests similar to those presented here and performed under identical conditions, is 300–500 μg./dog/day. This is 30–50 times more steroid than the 10 μg. daily requirement for maintaining adrenalectomized dogs with aldosterone.

Aldosterone in low dosage is less effective in regulating serum K levels than it is in maintaining a normal serum Na. In this respect it appears to differ from DOC, for aldosterone dogs receiving sub-maintenance doses and exhibiting severe symptoms of hyperpotassemia may present no detectable deviations from normal in serum Na. A larger dose of aldosterone is requisite to prevent a slow decline in arterial pressure than is necessary to keep the serum electrolyte pattern normal.

Aldosterone, although possessing certain physiological properties characteristic of DOC, also exhibits activity different from desoxycorticosterone and peculiar to itself.

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EFFECT OF ALCOHOL INTOXICATION AND ACTH ON LIVER ASCORBIC ACID IN THE GUINEA PIG[1,2]

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THE administration of an intoxicating amount of alcohol to intact rats (Forbes and Duncan, 1951; Smith, 1951) and guinea pigs (Forbes and Duncan, 1951) has been shown to cause a reduction in the concentration of ascorbic acid and cholesterol in the adrenal glands. This adrenal response does not occur in hypophysectomized rats (Forbes and Duncan, 1951; Smith, 1951). However, in spite of this effect of alcohol on the adrenal ascorbic acid, repeated moderate alcohol intoxication in guinea pigs maintained with a scorbutogenic diet did not appear to hasten the rate of development of scurvy (Forbes and Duncan, 1953). Mortality rate and adrenal cholesterol concentration both were quite comparable to those found in pair-fed controls. It would thus seem that the ascorbic acid that left the adrenals as a result of alcohol intoxication could not have been entirely lost to the body, but may have migrated to some other tissue or organ.

Loeper, (1936) and his associates induced severe phosphorus intoxication in guinea pigs for a period of 7 days and found a higher level of ascorbic acid in the liver of those animals than in corresponding controls whether the animals were maintained on a scorbutogenic diet or received daily injections of ascorbic acid. Dugal and Therien (1947) found that guinea pigs that had become adapted to exposure to severe cold for an extended period and had received vitamin C daily, tended to have a higher level of ascorbic acid in the liver than was found in controls receiving the same amount of the vitamin but kept at room temperature. Long (1946) reported no change in the liver ascorbic acid in guinea pigs 24 hours after a non-fatal hemorrhage. When a non-fatal hemorrhage was imposed on rats, which readily synthesize vitamin C, Sayers (1945) found a gradual but steady increase in the liver ascorbic acid at intervals up to 24 hours. More

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recently, Casentini and his co-workers (1953) studied the effect of various stressor agents on tissue ascorbic acid in guinea pigs and found a lower level in the liver of fasted animals subjected to a temperture of 0° to 4° C. for 40 hours than in fasted controls at room temperature.

In an attempt to obtain more information on the effect of alcohol on ascorbic acid in the body, it was decided to determine whether a change in the amount of ascorbic acid in the liver of guinea pigs might occur following alcohol intoxication.

PROCEDURE

Male guinea pigs weighing 250–300 gm. were placed on a diet of Rockland rabbit pellets plus an abundance of green cabbage leaves for 10–12 days in order to assure healthy animals in a fairly comparable nutritional condition. They were then paired by weight and all deprived of the green cabbage for various periods of time before they were given alcohol or used as controls. No more cabbage was fed during the course of an experiment. The alcohol was prepared as a 15% solution in 5% glucose and given by intraperitoneal injection at a level of 4 gm./kg. of body weight. Sterile technic was observed throughout. Controls were injected with a comparable volume of 5% glucose. A few animals were sacrificed without injections. Since the guinea pigs receiving alcohol rarely ate for the next 24 hours, no pellets were allowed any of the animals for this period after the first injection. All the alcohol-injected animals to be sacrificed later than 24 hours were given pellets *ad libitum* after this time. Their food consumption was checked several times during the day and the controls were given only the amount eaten by the corresponding test animals.

In one group of experiments the animals were sacrificed at intervals of 24, 48 and 72 hours after 1 injection of alcohol. In another series, alcohol was administered on 2 or 3 consecutive days. These guinea pigs were sacrificed 24 hours after the last injection, corresponding to 48 or 72 hours after the first intoxication. All animals were sacrificed and the adrenals removed and analysed for ascorbic acid according to procedures described in an earlier publication (Forbes and Duncan, 1951). The liver was removed, and a wedge-shaped piece weighing approximately 350–400 mg. was cut from the left lobe. The wedge was removed from the same location in each animal. This piece was weighed at once on a torsion balance, placed in 6% trichloracetic acid and analysed for ascorbic acid by the method used for the adrenals. The accuracy of using a single wedge-shaped piece from the liver was checked by determinations on whole livers and by wedges removed from other lobes of the same liver. The variations were never great.

Other groups received injections of 25 mg. of sodium ascorbate per 100 gm. of body weight 24 hours before the initial dose of alcohol or glucose and an additional 25 mg. per 100 gm. was added to the injected solutions.

RESULTS

The results obtained from these experiments are shown in Table 1. The values given are the mean and standard deviation for the total ascorbic acid in the fresh tissue of the liver and of the adrenals. The ascorbic acid concentration in the left adrenal was determined in each case and the total adrenal content calculated. It will be noted that 24 hours after 1 injection

TABLE 1. THE ASCORBIC ACID CONTENT OF THE LIVER AND ADRENALS OF GUINEA PIGS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF 4 GM. ALCOHOL/KG. OF BODY WEIGHT, PREPARED AS A 15% SOLUTION OF 95% ALCOHOL IN 5% GLUCOSE

Number of g.p. used	Solution given	Number of injections	Sacrificed		Ascorbic acid			
			After injection, hrs.*	Without greens, hrs.	Adrenals	Liver		
					Total mg.	Total mg.	mg./100 gm.	
					Mean SD	Mean SD	Mean SD	
6	—	—	—	64	0.186 ±0.076	1.63 ±0.53	0.39 ±0.11	
6	—	—	—	88	0.155 ±0.023	1.15 ±0.10	0.28 ±0.02	
6	—	—	—	112	0.119 ±0.034	1.01 ±0.27	0.25 ±0.07	
11	glucose	1	24	64	0.180 ±0.030	1.98 ±0.26	0.48 ±0.08	
11	alcohol	1	24	64	0.157 ±0.045	1.97 ±0.39	0.48 ±0.07	
10	glucose	1	48	64	0.146 ±0.036	1.29 ±0.21‡	0.36 ±0.05	
10	alcohol	1	48	64	0.145 ±0.031	1.82 ±0.44	0.50 ±0.11	
6	glucose	2	48	64	0.176 ±0.038	1.60 ±0.28	0.42 ±0.08	
7	alcohol	2	48	64	0.154 ±0.013	1.98 ±0.38	0.50 ±0.10	
8	glucose	1	48	112	0.161 ±0.047	1.04 ±0.15‡	0.24 ±0.03	
8	alcohol	1	48	112	0.117 ±0.011	1.62 ±0.29	0.38 ±0.06	
4	glucose	1	72	88	0.116 ±0.019	1.42 ±0.36	0.40 ±0.10	
4	alcohol	1	72	88	0.128 ±0.019	1.62 ±0.39	0.45 ±0.12	
8	glucose	3	72	88	0.155 ±0.029	1.28 ±0.12	0.31 ±0.02	
7	alcohol	3	72	88	0.105 ±0.010	1.46 ±0.20	0.36 ±0.06	
12†	glucose	1	24	64	0.301 ±0.061	4.30 ±1.20	1.11 ±0.32	
12	alcohol	1	24	64	0.219 ±0.043	3.98 ±0.67	1.00 ±0.13	
6†	glucose	1	48	88	0.225 ±0.043	2.05 ±0.37‡	0.52 ±0.11	
6	alcohol	1	48	88	0.244 ±0.054	2.88 ±0.61	0.73 ±0.14	

* Hours after first injection of glucose or alcohol.

† Animals in these groups received 2 injections of 25 mg. sodium ascorbate/100 gm. body wt., one 24 hrs. before alcohol or glucose, the other with injected solutions.

‡ This difference is considered statistically significant since the P value from Fisher's tables (1936) is less than 0.01.

of alcohol there was no difference between the liver ascorbic acid content of the alcohol and glucose-injected animals, although the expected drop in adrenal ascorbic acid had occurred. However, 48 hours after 1 injection of alcohol, a significantly higher level was found in the liver of the test animals than in the corresponding control groups which received glucose or were sacrificed after depletion only. The difference was still evident, but less marked, in the animals sacrificed 72 hours after 1 injection. This general effect on liver ascorbic acid was obtained even when additional alcohol was given on the intervening days. Administration of sodium ascorbate before and with the injected solutions raised the general level of ascorbic acid in both liver and adrenals; nevertheless, the livers of the alcohol-injected animals sacrificed at the 48-hour period contained more ascorbic acid than was found in the glucose controls. A 64-hour period of depletion

before the alcohol administration did not alter this relationship. Experimental results not shown in the Table indicated that when the total period of depletion was extended to 184 hours the difference between the test and control animals had disappeared.

Since alcohol intoxication had shown such a definite effect on the liver ascorbic acid, it was decided to determine whether ACTH might have a similar effect. Crystalline ACTH (Armour) was dissolved in 5% glucose so that 1 mg. or 1 International Unit was contained in 0.2 ml. of solution. This was given by intraperitoneal injection at dose levels of 2, 4, and 5 mg. per 100 gm. of body weight. The 5 mg. dose was divided into 2 injections, the second following the first by 12 hours. Since the ACTH exerted no apparent effect on food intake, all animals were allowed pellets *ad libitum*. They were sacrificed at intervals of 12, 24, 36, and 48 hours after the injection. The values for all dose levels have been combined and are presented in Table 2. The results show that the livers of the ACTH-injected groups

TABLE 2. THE ASCORBIC ACID CONTENT OF THE LIVER AND ADRENALS OF GUINEA PIGS FOLLOWING 1 OR 2 INTRAPERITONEAL INJECTIONS OF ACTH

Number of g.p.	Substance given	Sacrificed		Ascorbic acid			
		After inj., hrs.	Without greens, hrs.	Adrenals	Liver		
				Total mg.	Total mg.	mg./100 gm.	
				Mean SD	Mean SD	Mean SD	
14	Glucose	24–36	40	0.197 ±0.037	2.11 ±0.54	0.53 ±0.13	
15	ACTH*	24–36	40	0.159 ±0.049	2.72 ±0.58	0.67 ±0.14	
14	Glucose	48	64	0.151 ±0.025	1.75 ±0.39	0.46 ±0.10	
15	ACTH*	48	64	0.159 ±0.048	1.83 ±0.56	0.50 ±0.15	

* ACTH given at 3 dose levels; 2, 4, and 5 mg./100 gm. body wt. Values for all doses combined. Prepared so 1 mg. crystalline ACTH contained in 0.2 ml. 5% glucose, controls, 5% glucose.

sacrificed 24 or 36 hours after injection contained more ascorbic acid than was found in the glucose controls. The mean value at these intervals was significantly different from the mean of the controls, the *P* value being less than 0.01. The difference by 48 hours was not significant.

DISCUSSION

These experiments demonstrate the occurrence of a significantly higher content of ascorbic acid in the liver of guinea pigs 48 hours after alcohol intoxication than was found in comparable controls. This effect was not altered by a second injection of alcohol on the intervening day before sacrifice, or by the injection of a large amount of sodium ascorbate before and with the alcohol. Moderate vitamin C depletion before the alcohol did not prevent the occurrence of this higher level, but when the total period of

vitamin depletion was extended to 184 hours there was no significant difference between the test and control animals. This is probably due to the generally low level of ascorbic acid in the animal at this time. It is of interest that the alcohol animals sacrificed after a total depletion of 112 hours showed an average value comparable with that seen in the uninjected controls after 64 hours depletion. Since the guinea pig is believed to be unable to synthesize vitamin C, this higher level in the animals injected with alcohol or ACTH probably represents migration from other areas of the body into the liver. This extra amount of vitamin C in the liver of the test animals could not have come entirely from the adrenals since the adrenal loss represents only a small fraction of the difference seen in the liver. Furthermore, the maximal difference in the liver occurred in the alcohol guinea pigs well past the usual period of depletion of the adrenal ascorbic acid. At the time of the greatest drop in the adrenal, the liver showed no change. These results suggest that the lack of any difference in the liver ascorbic acid which was noted by Long (1946) in guinea pigs following nonfatal hemorrhage may have been due to the fact that the animals were sacrificed too soon.

Selye (1950) pointed out that the imposition of a stress instigates a series of metabolic changes which seem to progress according to the type and severity of the stress, and which are generally reversed during the stage of resistance. In our animals injected with ACTH, both the liver and adrenals respond about 24 hours earlier than in the alcohol-injected guinea pigs. This is not surprising since the injection of ACTH would be expected to have a more acute and less persistent effect than alcohol intoxication. Casentini and his associates (1953) sacrificed their animals while they were still subjected to both cold and fasting, and found a moderate reduction in liver ascorbic acid. It is possible that if we had sacrificed some of our alcohol-injected animals earlier than 24 hours they might have shown a lower value than the controls. Since the higher level occurred a relatively long time after the imposition of the stress, it is suggested that mobilization of ascorbic acid into the liver may be a part of the adaptation mechanism.

SUMMARY

Guinea pigs given an intraperitoneal injection of an intoxicating amount of alcohol on 1 to 3 consecutive days showed significantly higher values for total liver ascorbic acid 48 hours after the initial intoxication than were found in controls receiving diluting fluid only. This occurred whether the guinea pigs had received large amounts of sodium ascorbate or not. The difference in liver ascorbic acid had not developed 24 hours after the initial intoxication but was still apparent at 72 hours. A similar higher level in the

liver ascorbic acid was found after injection of ACTH, although this occurred approximately 24 hours sooner than was the case with alcohol.

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DEPENDENCE OF RATIO OF RADIOIODIDE CONCENTRATIONS IN THYROID GLAND AND SERUM ON SERUM IODIDE CONCENTRATION: WITH PROPYLTHIOURACIL

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A STUDY of the dependence of the ratio of the thyroid radioiodide concentration to the serum radioiodide concentration (T/S ratio) on the serum iodide concentration has been reported in the rat by Halmi (1954). This paper reports a similar study in normal and hypophysectomized mice. In addition, using simple models, formulae have been derived describing the dependence of the T/S ratio on the serum iodide concentration and predictions of the formulae are compared with the appropriate experimental results in both mice and rats.

MATERIALS AND METHODS

Male C_3H mice were used. The normal mice were four months of age at the time of the radioiodine study. The hypophysectomized mice were fourteen weeks old at the time of the operation which was done eight days before the radioiodine study. They were hypophysectomized through the parapharyngeal approach. Completeness of the operation was ascertained by visual inspection of the region of the sella turcica for pituitary fragments using a binocular microscope at 9× magnification.

The normals were fed a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner and Morris, 1953) for 35 days and the hypophysectomized mice for 22 days immediately preceding the radioiodine study.

Organic binding of iodine was blocked by subcutaneous injection of a solution containing 2.5 mg. of propylthiouracil (Wollman and Scow, 1953).

Administration of iodide: A different stock aqueous solution of NaI was used for each of the two experiments reported. The iodide concentration was measured by direct titration or by a micro-Volhard method depending upon the iodide concentration.[2] Radioiodide[3] was added to appropriate dilutions of the stock NaI solutions. One-half ml. of a solution of known specific activity, containing between 20 to 100 microcuries of radioiodide was administered by intraperitoneal injection to each mouse one hour after the propylthiouracil injection.

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[2] We are grateful to R. Koegel of the National Cancer Institute for analyses of the stock iodide solutions.

[3] The I^{131} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Thyroid gland and blood samples were obtained as previously described by Wollman and Scow (1953) one hour after radioiodide injection for normals, and two hours after injection for hypophysectomized mice.

Radioiodide content of samples was measured using well-type gamma counters. The more active samples were measured using the 4-wire counter previously described (Wollman, Morris and Green, 1951), and the less active samples using a Texas Company type high efficiency Geiger tube.[4]

Stable iodide concentrations in thyroid glands and serum were obtained from the radioiodine measurements and the known specific activities of the injected solutions. This method measures only freely-exchangeable thyroid iodide. No evidence is available as yet that any other thyroid iodide fraction exists under the conditions of these experiments (Wollman and Scow, 1953). The error due to neglecting the endogenous serum iodide concentration was negligible except in the case of the lowest dose of iodide to the hypophysectomized mice.

Nature of the thyroidal I^{131}: The thyroid gland of a normal mouse which was given propylthiouracil and 100 microcuries of carrier-free radioiodide was removed one hour after radioiodide injection. After homogenization in 1 ml of ice-cold H_2O containing 10 μg. NaI, the suspension was deproteinized with 0.1 ml each of Somogyi's $Ba(OH)_2$ and $ZnSO_4$ solutions. After centrifugation the amount of I^{131} in the supernate and in the precipitate were determined. One-tenth and two-tenths ml of the supernate (in 0.01 ml. aliquots) were then put on Whatman's No. 1 filter paper, and the amount of I^{131} put on paper was measured. The filter paper was developed by either of two chromatographic solutions: a. 81 parts 95% ethanol—19 parts 2N NH_4OH; or b. the upper phase obtained by shaking 10 parts n-butanol, 1 part glacial acetic acid and 5 parts water. Chromatographs were run for 16 hours by the ascending method of Williams and Kirby (1948). Localization of radioactive spots was ascertained by autoradiographs of the paper using Kodak No-Screen X-Ray film. Radioactive spots were cut out and I^{131} was determined in the spots and in the remainder of the strip by gamma counting in order to check recovery of I^{131} put on paper.

RESULTS

The I^{131} was in the form of iodide in thyroid glands in mice given 2.5 mg. of propylthiouracil before the radioiodide injection. Almost all of the I^{131} was found in the supernate after deproteinization of homogenates of the thyroid gland, and the only significant localization of the I^{131} on the paper chromatogram was at the site of iodide (Fig. 1). With ethanol/ammonium hydroxide almost all I^{131} put on the paper was recovered in the iodide spot. With n-butanol acetic acid, however, only 64% was recovered at the iodide spot; 15% was distributed uniformly between the iodide spot and the solvent front; and 21% was missing. The missing I^{131} may have evaporated from the paper.

The T/S ratio was relatively independent of the serum iodide concentration for low serum iodide concentrations, but it decreased with increasing serum iodide concentration at higher serum levels in both normal and hy-

[4] Available from Welch Allyn, Inc., Skaneateles Falls, New York.

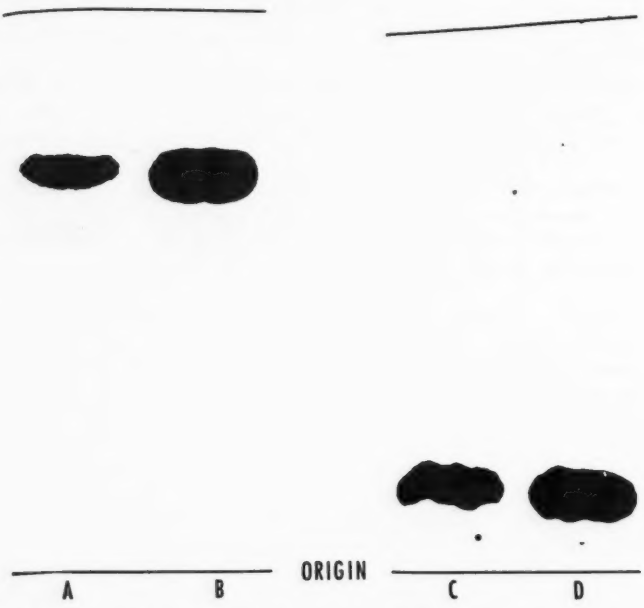

FIG. 1. Autoradiographs of paper chromatographs of supernates of homogenates of mouse thyroids deproteinized with $Ba(OH)_2$-$ZnSO_4$. The mice had been treated with 2.5 mg. of propylthiouracil to block organic binding of I^{131}. A and B: Ethanol/ammonium hydroxide. A: Supernate. B: Standard radioiodide. C and D: n-butanol/acetic acid/water. C: Supernate. D: Standard radioiodide.

pophysectomized mice (Table 1 and Fig. 2). In order to determine whether the decrease in the T/S ratio was due to an increased time interval required for equilibration of iodide between blood and thyroid gland in nor-

TABLE 1. EFFECT OF SERUM IODIDE CONCENTRATION ON T/S RATIO FOLLOWING A SINGLE INJECTION OF PROPYLTHIOURACIL IN NORMAL AND HYPOPHYSECTOMIZED MICE

Group	Dose iodide, μg.	Number mice	Mouse weight, gm.	Thyroid weight, mg.	T/S	Thyroid iodide, mg./100 gm.	Serum iodide, μg./100 ml.	Per cent of I^{131} dose per ml. serum
Normal	0	5	30.0±2.1[1]	2.1±0.2[1]	301 ±11[2]			5.76±0.45[2]
	8.98	5	28.4±1.3	2.1±0.2	295 ±13	13.6 ±0.8[2]	46 ± 1.4[2]	4.86±0.25
	44.9	5	29.6±1.7	2.2±0.3	124 ± 6	32.5 ±3.0	262 ± 18	5.75±0.28
	180	5	28.8±1.7	2.2±0.3	68.3 ± 3.2	61.8 ±3.8	906 ± 44	4.85±0.33
	898	5	27.8±0.9	1.9±0.3	14.3 ± 1.2	76.8 ±3.0	5470 ± 420	5.62±0.39
	8980	5	27.0±1.7	2.2±0.3	2.06± 0.14	92.8 ±1.5	45600 ±3400	4.53±0.20
Hypophysectomized	0	4	21.5±1.5	1.5±0.3	79.6 ±10.1	—	—	4.98±0.35
	0.816	5	20.2±2.1	1.7±0.2	67.8 ± 9.4	0.33±0.09	6.26± 0.95	5.05±0.58
	4.08	4	21.5±1.5	1.9±0.2	59.3 ± 3.3	1.09±0.43	31.0 ± 2.6	5.37±0.22
	20.4	5	21.6±1.3	1.5±0.2	42.2 ± 4.1	3.55±0.39	151 ± 7	5.30±0.16
	102.1	4	22.3±1.9	1.5±0.2	19.0 ± 1.4	7.72±0.62	788 ±34	5.70±0.18

[1] Mean±standard deviation.
[2] Mean±standard error.

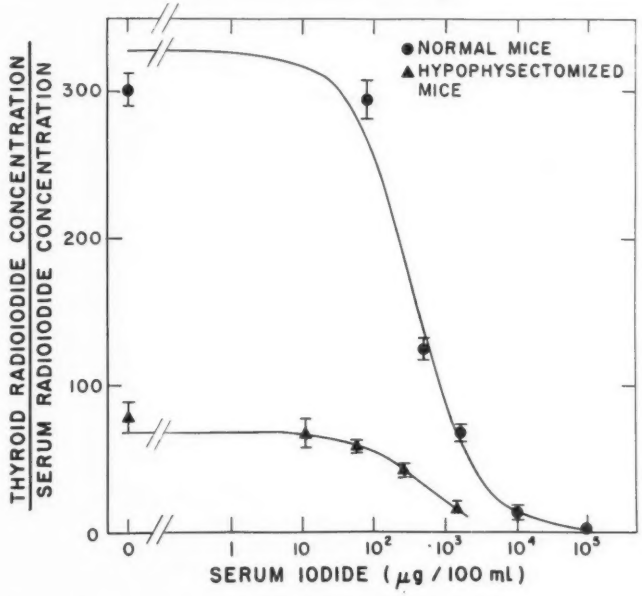

FIG. 2. Dependence of the ratio of thyroid radioiodide concentration to serum radioiodide concentration on serum iodide concentration. The points are experimental data, the vertical line through each point is the standard error. The smooth curves represent least squares fit of $T/S = (A/K_M + I_B) + 1$ to the experimental data.

mal mice, a comparison was made of the T/S ratio at one and two hours after an injection of a radioiodide solution containing approximately 50 micrograms of iodide. The T/S ratio was found to be the same at both time intervals. The relation between the concentration of freely-exchangeable thyroid iodide and the serum iodide concentration is shown in Figure 3.

MODELS

To interpret the above results, equations have been derived which describe the dependence of the T/S ratio on the serum iodide concentration. The derivations have been based on two qualitatively different models (Means, 1951):

1. *Iodide concentrating as an adsorption process:* In this physicochemical model, the thyroid gland is assumed to contain a substance or adsorber which complexes with iodide. The adsorber is to exist at a concentration of M moles of adsorption sites per gram of thyroid. In addition to adsorbed iodide, free iodide is also assumed to exist in the thyroid at a concentration equal to that in the blood, I_B. The amount of adsorbed iodide is assumed to be governed by the law of mass action or its equivalent, the Langmuir ad-

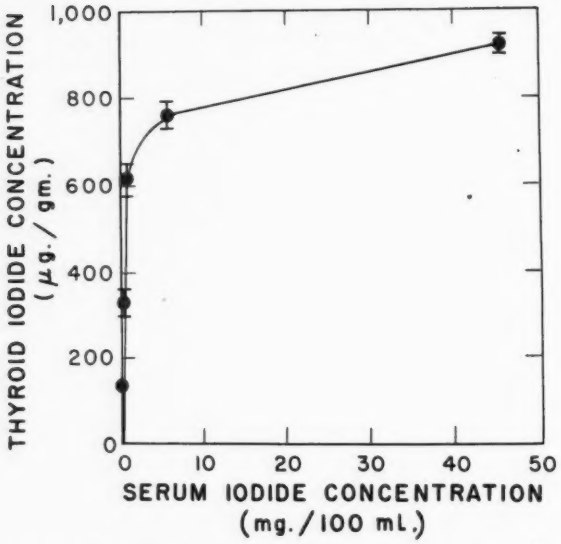

FIG. 3. The relation between thyroid iodide concentration and serum iodide concentration in normal mice. The vertical line through each point represents standard error of the mean.

sorption isotherm (de Boer, 1953). That is, the rate of adsorption, v_1, is proportional to the product of the concentration of free iodide, I_B, and the concentration of unoccupied adsorption sites, $M - I_T'$, where I_T' is the concentration of adsorbed iodide. The rate of desorption, v_2, is proportional to the concentration of adsorbed iodide. The assumptions may be written

$$v_1 = k_1(M - I_T')I_B$$

$$v_2 = k_2 I_T'.$$

At equilibrium $v_1 = v_2$, and hence

$$\frac{k_2}{k_1} = \frac{(M - I_T')I_B}{I_T'} = K_M \qquad (1)$$

where K_M is the equilibrium constant for iodide-adsorber complex formation. On solving eq. (1) for I_T' this yields

$$I_T' = \frac{MI_B}{K_M + I_B}$$

To obtain the total thyroid iodide concentration, I_T, the free thyroid iodide concentration which is assumed to equal the blood iodide concentration I_B, must be added to I_T'. This yields after dividing through by I_B

$$\frac{I_T}{I_B} = \frac{M}{K_M + I_B} + 1. \tag{2}$$

2. *Iodide concentrating as an active transport process:* In the simplest form of this model the concentrated iodide is assumed to be localized in regions surrounded by continuous membranes (consisting either of cells or cell membranes) which separate the concentrated iodide from non-concentrated iodide. To quantitate this model[5] the iodide is assumed to complex with a transport carrier molecule localized in the membrane where M is the concentration of transport carrier. Iodide is assumed to enter the region of concentrated iodide by active transport with entry rate proportional to the concentration of iodide-carrier complex, and in addition by diffusion at a rate proportional to the iodide concentration, I_B, outside the region of concentrated iodide. That is, the total rate of entry, v_1, is given by

$$v_1 = K_T[I_B \cdot M] + K_d I_B$$

where K_T is the rate constant for transfer of complexed iodide, $[I_B \cdot M]$ is the concentration of complexed iodide, and K_d is the rate constant describing the diffusion of iodide into the region of concentrated iodide. The concentration of iodide complex is assumed to be governed by the law of mass action with an equilibrium constant, K_M, given by

$$K_M = \frac{I_B(M - [I_B \cdot M])}{[I_B \cdot M]} \tag{3}$$

where, as before M is the concentration of complexing agent (transport carrier). The rate of loss of concentrated iodide from the region of concentrated iodide is assumed to be proportional to the concentration of the iodide in the region, I_R, that is

$$v_2 = K_d I_R.$$

At equilibrium, $v_1 = v_2$, and using the expression for $[I_B \cdot M]$ obtained from eq. (3) the relation between I_R and I_B is found to be

$$I_R = \frac{K_T M/K_d}{K_M + I_B} I_B + I_B.$$

The average thyroid iodide concentration, I_T, is found by

$$I_T = F_R I_R + (1 - F_R) I_B$$

where F_R is the fraction of the thyroid volume occupied by regions of concentrated iodide and where it is assumed that the thyroid iodide concen-

[5] Karreman (1953) has made detailed analysis of the implications of these assumptions.

tration outside such regions is the same as the blood iodide concentration, I_B.

Hence

$$\frac{I_T}{I_B} = \frac{F_R K_T M / K_d}{K_M + I_B} + 1 \tag{4}$$

Both the adsorption and the active transport models yield equations of the same form

$$T/S = \frac{I_T}{I_B} = \frac{A}{K_M + I_B} + 1 \tag{5}$$

where A is a constant whose significance depends upon the model, and K_M is an equilibrium constant. The present data cannot distinguish between the models. Although both models involve the formation of a complex between iodide and a complexing agent, one fundamental difference between the models exists. In the adsorption model the concentrated iodide is complexed whereas in the active transport model the concentrated iodide is free and the iodide complex is in equilibrium with the low concentration of iodide existing in the blood rather than with the high concentration existing in regions of concentrated iodide. This and other factors to be discussed in another paper may allow selection of the correct model.

Using the method of least squares eq. (5) was fit to the data for both normal and hypophysectomized mice (Fig. 2). Values of the constants A and K_M obtained from data in this paper, and from the data on rats obtained by Halmi (1954) are listed in Table 2. The theoretical equation fits

TABLE 2. BEST VALUES OF CONSTANTS OBTAINED FROM LEAST SQUARES FIT OF $T/S = (A/K_M + I_B) + 1$ TO DATA ON MICE AND RATS

Species	Group	A	K_M, μg./ml.
Mouse	Normal	67	0.205 ±0.01[1]
	Hypophysectomized	16	0.248 ±0.03
Rat[2]	Propylthiouracil	66.6	0.60 ±0.06
	Normal	7.37	0.31 ±0.06
	Hypophysectomized[3]	2.83	0.58 ±0.16

[1] Standard error.
[2] Halmi (1954).
[3] $T/S = (A/K_M + I_B) + C$.

the experimental data well. However, there are small but statistically significant deviations of the data from the equations which may reflect one or more of the following discrepancies between the model and the actual gland:

1. The concentration of iodide has been used instead of its chemical activity.

2. The serum iodide concentration has been used instead of the free iodide concentration in the cell-free part of the blood. These concentrations probably differ only by a small factor, but their ratio may vary with the blood iodide concentration. The extent to which blood iodide is complexed with blood proteins does not appear to be known.

3. The electric charge on the iodide has been ignored. A difference in electric potential between the capillaries and the site of iodide concentration, perhaps between the inside and outside of the membrane surrounding a region of concentrated iodide, would be expected to influence iodide in a manner qualitatively similar to chloride, i.e., partially exclude it. Because of this effect the assumption that the thyroid iodide concentration outside of regions of concentrated iodide is the same as that of the blood may be incorrect. As a consequence the constant term in eq. (5) cannot be expected to be unity but less than unity. The discrepancy due to this effect would be expected to be most apparent at high serum iodide concentrations where the T/S ratio is approximately one or less. A large literature exists describing the influence of the ionic charge on ion transport (Conway, 1953; Teorell, 1953).

4. All the regions of concentrated iodide have been assumed to have the same T/S ratio. Autoradiographic studies of the localization of radioiodide in the rat by Pitt-Rivers and Trotter (1953) and in the mouse (Wollman and Wodinsky, unpublished results) show that the T/S ratio differs from follicle to follicle. Since $K_M + I_B$ may be considered to be constant for all follicles in a gland, A measures an average value of $K_T M/K_d$ (eq. 4 and 5).

DISCUSSION

The general form of eq. (5) describing the dependence of the T/S ratio on the serum iodide concentration

$$T/S = \frac{A}{K_M + I_B} + C$$

fits the experimental data satisfactorily over the entire range of serum iodide concentrations. The constants A, K_M and C may be obtained by inspection of a plot of T/S vs. I_B. K_M is equal to the value of I_B when $T/S - C$ is half that when $I_B = 0$.

The constant, K_M, has the same interpretation with either model. It is a measure of the affinity of a particular substance in the thyroid gland for iodide. Since K_M has the same value in normal and hypophysectomized mice it might be concluded that removal of the pituitary does not affect the affinity of the substance for iodide. K_M may therefore be expected to be the same for a given strain of animal in all states of thyroid activity. Although the value of K_M does not appear to be the same for normal and

propylthiouracil-treated rats (Halmi, 1954) the values estimated from the data may not be correct. The value of K_M is sensitive to small systematic errors in the measurements, and the experimental points were not spaced properly to allow an accurate determination of K_M in these rats. If, in the rat, K_M is constant and larger than the value in the mouse, as suggested by the data in Table 2, it will imply that the substance complexing with iodide in the mouse thyroid is different from that in the rat, and has a greater affinity for iodide than that in the rat.

The mechanism by which hypophysectomy or propylthiouracil feeding affects the T/S ratio is not significantly clarified by the present analysis even in the event that K_M turns out to be a constant for the strain or species of animal. In the adsorption model the T/S ratio can be affected only by the quantity of adsorber. In the active transport model, however, the T/S ratio might be affected through changes in 1) the fraction of the thyroid volume occupied by regions of concentrated iodide, 2) the concentration of transport carrier, 3) the rate of transport of complexed iodide, 4) the rate of diffusion out of the regions of concentrated iodide.

SUMMARY

The dependence of the ratio of the iodide concentrations in thyroid gland and serum (T/S ratio) on the serum iodide concentration was investigated in normal and hypophysectomized C_3H mice. Equations derived on the basis of an adsorption model or an active transport model are of the same form, and predict the observed dependence over almost the entire serum iodide concentration range.

Acknowledgment

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DEPENDENCE OF RATIO OF RADIOIODIDE CONCENTRATIONS IN THYROID GLAND AND SERUM ON SERUM IODIDE CONCENTRATION: WITHOUT PROPYLTHIOURACIL

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THE ratio of the thyroid radioiodide concentration to the serum radioiodide concentration (T/S ratio) was less when organic binding of iodine by the thyroid gland was permitted, than when binding was blocked by propylthiouracil (Wollman and Scow, 1953). Since a sufficiently high concentration of serum iodide is known to block binding of iodine in the rat (Wolff and Chaikoff, 1948), it might be expected to block thyroid binding of iodide in the mouse and thereby elevate the T/S ratio in the mouse.

This paper presents data describing the dependence of the T/S ratio on the serum iodide concentration in mice. The preceding paper (Wollman and Scow, 1954) described a similar study with the exception that the mice were given a single dose of propylthiouracil to block binding. A comparison is made of the T/S ratio when binding is blocked by iodide with that when binding is blocked by both propylthiouracil and iodide.

METHODS AND MATERIALS

Male C3H mice, four months of age, were used. They formed a duplicate group to the normals used in the preceding paper (Wollman and Scow, 1954). They had been fed a pelleted diet of moderately low iodine content for 34 days prior to the radioiodine study (Wollman, Scow, Wagner and Morris, 1953).

Stable iodide (NaI) and radioiodide[2] were injected in a solution of known specific activity as described previously (Wollman and Scow, 1954). One hour after iodide injection, mice were anesthetized with ether and a blood sample was obtained from the heart. Thyroids were then immediately removed, cooled, weighed and homogenized in 6 ml. of ice-cold H_2O containing 1 mg. of NaI. The homogenate was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ as described previously (Wollman and Scow, 1953). I^{131} was measured in precipitated and non-precipitated fractions of the homogenate and in serum as described in the preceding paper (Wollman and Scow, 1954). The amount of stable freely-

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[1] National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[2] The carrier-free I^{131} used in this study was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

exchangeable iodide in the thyroid gland and in serum and the amount of stable iodine bound during the interval between injection and death were determined using the known specific activity of the injected iodide solutions.

Identification of I^{131} containing compounds in the supernatant: One and two hours after radioiodide injection, thyroid glands were excised from mice, cooled, weighed and then homogenized in 1 ml. of ice-cold H_2O containing 10 μg. of NaI. The homogenate was deproteinized as above, and I^{131} was measured in the supernatant and the sediment. Five micrograms of diiodotyrosine were added to each supernatant. Aliquots of the supernatants then were put on filter paper and developed using either ethanol/NH_4OH or n-butanol/acetic acid/H_2O and one dimensional paper chromatography. Localization of radioactivity and measurement of I^{131} in spots as well as recovery checks were done as described in the preceding paper (Wollman and Scow, 1954).

RESULTS

Almost all of the I^{131} in the deproteinized homogenates was in the form of iodide (Fig. 1 and Table 1). Using the data in Table 1, it is possible to estimate that over 99.9% of the organic radioiodine was removed from the homogenate by deproteinization with $Ba(OH)_2$ and $ZnSO_4$.

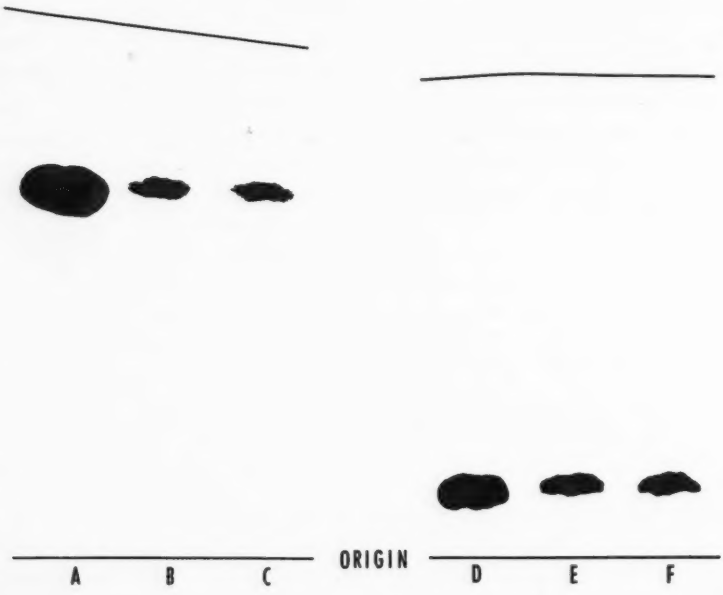

FIG. 1. Autoradiographs of chromatographs of supernatants of homogenates of mouse thyroid glands deproteinized by $Ba(OH)_2$-$ZnSO_4$. A, B, C: Ethanol/2N NH_4OH; A. Standard radioiodide; B. One hour after injection of radioiodide; C. Two hours after injection of radioiodide. D, E, F: n-butanol/acetic acid/H_2O; D. Standard radioiodide; E. One hour after injection of radioiodide; F. Two hours after injection of radioiodide.

TABLE 1. DISTRIBUTION OF I^{131} IN HOMOGENATES OF MOUSE THYROID GLAND

Treatment	Counts per second		Per cent of supernatant aliquot on paper						
	Supernatant	Sediment	Ethanol/NH_4OH			n-butanol/acetic acid/H_2O			
			Origin	Iodide	Rest	Origin	Iodide	Rest	Missing
1 hr. post injection	23.3	194	0	95	0	0	68	11	21
2 hrs. post injection	23.6	752	2	103	5	2	57	16	25
Propylthiouracil, 1 hr. post I^{131} injection	44.3	5.03	0	95	0	0	60	15	25

The dependence of the T/S ratio and the binding of iodine on the serum iodide concentration is shown in Table 2 and Fig. 2. The T/S ratio was found to be constant as the serum iodide concentration increased until the per cent of the dose which was organic bound began to decrease. The T/S ratio then increased progressively until the per cent of the administered radioiodide which was bound became very small. At higher concentrations of serum iodide, the T/S ratio decreased progressively as the serum iodide concentration increased. Although the T/S ratio decreased, the stable thyroid iodide concentration increased as the serum iodide concentration increased (Fig. 3).

A comparison of the dependence of the T/S ratio on serum iodide concentration when binding was blocked by iodide with the corresponding data when binding was blocked with propylthiouracil and iodide (Fig. 4) indi-

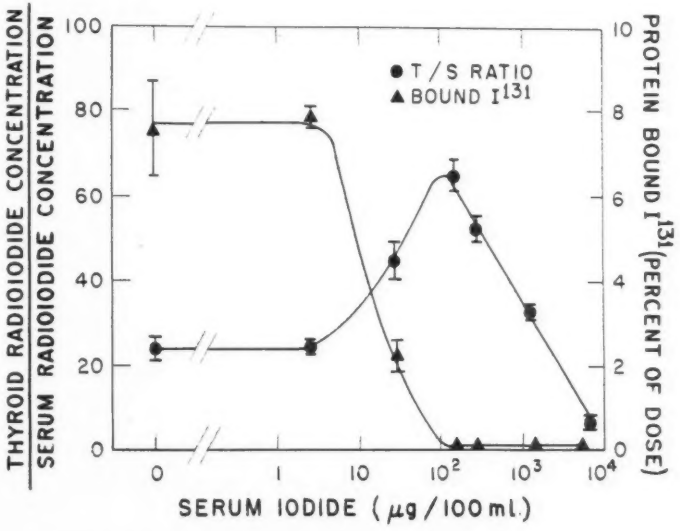

FIG. 2. The dependence of T/S ratio and per cent of administered radioiodide incorporated into protein binding in one hour on serum iodide concentration. The vertical line through each point represents standard error of the mean.

TABLE 2. EFFECT OF SERUM IODIDE CONCENTRATION ON THE T/S RATIO AND ON BINDING OF IODINE IN THE THYROID GLAND OF NORMAL MICE[1]

Dose iodide, μg.	Mouse weight, gm.	Thyroid weight, mg.	T/S	Thyroid iodide, μg./100 gm.	Serum iodide, μg./100 ml.	Per cent of I^{131} dose bound[4]	Iodine bound in 1 μg. hr.	Per cent of I^{131} dose per ml. serum[5]
0	30.0±4.7[3]	2.1±0.4[2]	24.3 ±2.1[3]	—	—	7.62 ±1.2[3]	—	5.87±0.30[3]
0.449	28.0±2.4	2.3±0.4	24.9 ±1.2	67± 4[3]	2.70± 0.17[3]	7.79 ±0.23	0.0358 ±0.0011[3]	5.54±0.15
4.49	30.5±2.8	2.3±0.5	45.6 ±4.1	1,290± 120	28.5 ± 2.2	2.25 ±0.35	0.101 ±0.016	6.38±0.36
22.5	29.7±3.2	2.3±0.4	65.8 ±3.6	10,100± 600	155 ± 10	0.076 ±0.004	0.0172 ±0.0009	6.70±0.22
44.9	28.5±2.4	2.2±0.4	53.3 ±2.6	14,600± 900	276 ± 21	0.0279 ±0.0023	0.0126 ±0.0010	5.78±0.29
179.6	30.2±3.6	2.4±0.5	33.4 ±1.7	43,200±2,900	1,300 ± 70	0.0146 ±0.0020	0.0262[6]±0.0036	7.17±0.15
898	28.3±0.4	2.2±0.2	7.44±0.49	52,200±2,300	7,070 ±280	0.00298 ±0.00028	0.0267[6]±0.0025	7.45±0.29

[1] Six mice per group.
[2] Standard deviation.
[3] Standard error of the mean.
[4] One hour after radioiodide injection.
[5] Corrected to 30 gm body weight.
[6] Overestimates due to contamination by iodide.

cated that the T/S ratio was approximately twice as large when the propylthiouracil was given. This result does not appear to be due to an incomplete equilibration of iodide between thyroid gland and blood in the absence of propylthiouracil since a test showed that the T/S ratio was the same at one hour as at two hours after radioiodide injection.

DISCUSSION

The curve describing the dependence of the T/S ratio on the serum iodide concentration (Fig. 2) appears to have the following qualitative inter-

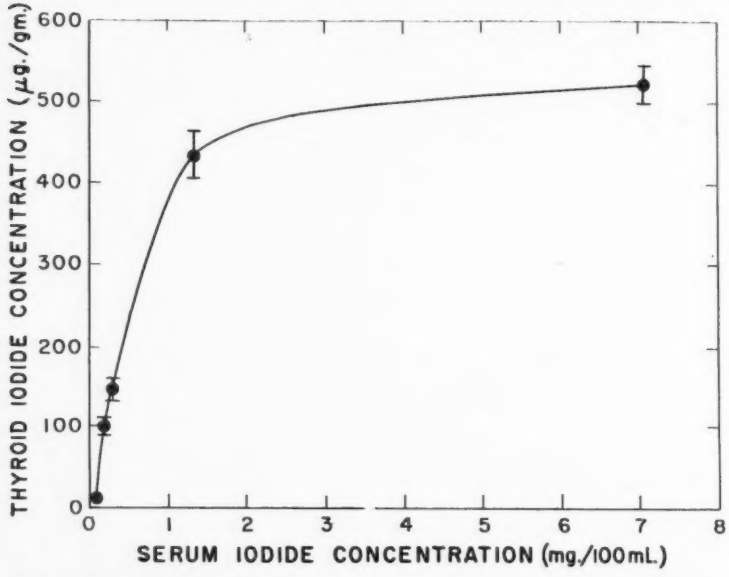

FIG. 3. The dependence of thyroidal iodide concentration on serum iodide concentration. The vertical line through each point represents standard error of the mean.

pretation: The T/S ratio is independent of the serum iodide concentration until the corresponding thyroid iodide concentration is high enough to begin to saturate the binding mechanism. The T/S ratio increases with increasing serum iodide concentration as the saturation becomes more complete. When the binding process is able to remove only a negligible fraction of available iodide, the T/S ratio then begins to fall with increasing serum iodide concentration. This occurs because the iodide concentrating mechanism is becoming increasingly more saturated as in the case of the propylthiouracil blocked thyroid at the same concentration range of serum iodide (Wollman and Scow, 1954).

When organic binding was blocked by high iodide levels, the value of the T/S ratio for a given serum iodide concentration was always lower than that in corresponding mice given propylthiouracil (Fig. 4). This observation may be considered evidence that propylthiouracil affects the T/S ratio by some mechanism in addition to the blocking of binding of iodide.

A sufficiently high concentration of serum iodide or thyroid iodide was associated with a decrease in the uptake of radioiodine. Although the uptake of radioiodine decreased to approximately one-third of normal when 4.49 μg. of iodide was given (Table 2) the amount of iodine bound in one

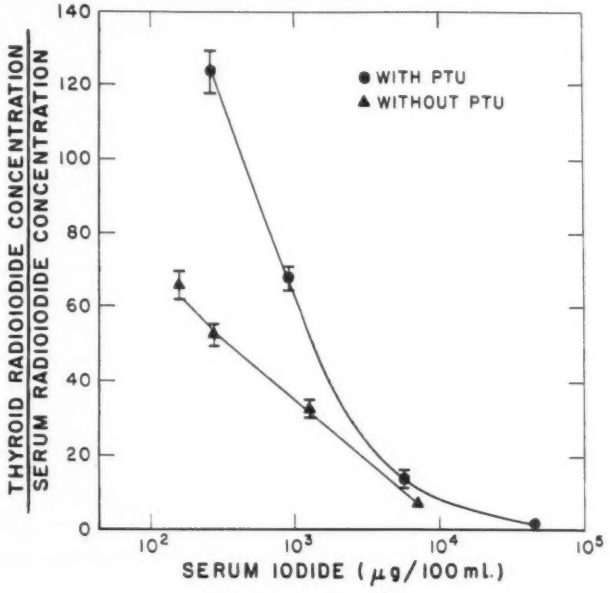

FIG. 4. The effect of propylthiouracil on the T/S ratio at various concentrations of serum iodide. The vertical line through each point represents the standard error of the mean.

hour increased indicating that a partial saturation of the binding mechanism was responsible for this decrease in uptake. However, when 22.5 μg. of iodide was given, not only was the uptake of radioiodide into thyroid protein decreased but the amount of iodine bound at one hour was decreased to one-sixth of that when 4.49 μg. of iodide was given (Table 2). This indicates a real inhibition of the binding mechanism rather than a saturation under these conditions. A similar inhibition has been observed in the rat (Wolff and Chaikoff, 1948; Raben, 1949).

The mechanism by which a high thyroid iodide concentration affects the binding process is unknown. Almost no information is available on the number or the nature of the steps involved in the binding process and on whether they are enzymatic or non-enzymatic. Consequently, it is difficult to determine whether high iodide concentrations affect binding by saturating or inhibiting enzymes, or by interfering with one or more non-enzymatic processes.

In the last few years two non-enzymatic mechanisms have been suggested by which a high iodide concentration might affect the binding process. Both mechanisms are based upon an analysis by Li (1942) of the kinetics of the non-enzymatic iodination of tyrosine.

The more recent suggestion (Fawcett and Kirkwood, 1953) is that iodide inhibits the binding of radioiodine by forming an I_3^- complex with free I_2, thus preventing iodination by I_2. This mechanism does not appear to be able to account for the saturation as shown by the following calculation[3]: At a thyroid iodide concentration of 1.29 mg. per 100 gm. thyroid (10^{-4}M) the I^{131} bound is only 30% of that when no carrier is given. At this iodide concentration (10^{-4}M) only a negligible fraction of the inorganic I_2 can be expected to be complexed since using the equilibrium constant for the formation of I_3^- at 38° C. (Davies and Gwynne, 1952)

$$\frac{[I^-][I_2]}{[I_3^-]} = 10^{-4}\,\frac{[I_2]}{[I_3^-]} = 0.00170$$

or

$$[I_2]/[I_3^-] = 17.$$

Although it appears unlikely that the formation of I_3^- can account completely for the inhibition of binding this possibility cannot yet be excluded. Using 10.1 mg. iodide per 100 gm. thyroid (0.8×10^{-3}M) as the concentration of thyroid iodide at which the iodination process is inhibited (Table 2) $[I_2]/[I_3^-]=2$. Hence two-thirds of the iodine in the thyroid can be expected

[3] In the following calculations the iodide concentrations used are those for freely-exchangeable iodide. If thyroid iodide exists which is not freely-exchangeable, it is assumed not to take part in the inhibition of the binding process by iodide.

not to be complexed with iodide. Under these circumstances it might be expected that the iodine bound would be reduced to two-thirds whereas actually it was reduced to one-sixth of that in the saturation region just discussed. However, if the local iodide concentrations at the binding sites were twice the average thyroid iodide concentration, complexing might account for most of the inhibition.

The older suggestion (Harington, 1951) is that thyroglobulin is iodinated by HIO rather than by I_2, that the HIO is formed by $I_2+H_2O \rightleftarrows H^+ + I^- +$ HIO and that the concentration of HIO would be expected to be decreased at high iodide concentrations. At a thyroid concentration of iodide sufficient to inhibit the binding process the extent of the reaction is indeed decreased, for according to Latimer (1951)

$$\frac{[H^+][I^-][HIO]}{[I_2]} = 3 \times 10^{-13}.$$

If the pH = 7 and $[I^-] = 10^{-3}$M, $[HIO]/[I_2] = 0.003$. However, the extent of the reaction varies inversely as the iodide concentration so that this mechanism might account for the drop in the binding of iodine if the extent of formation of HIO were rate limiting. It appears difficult to evaluate the quantitative significance of both the above suggestions since as evidenced by Ingold's (1953) discussion, even the non-enzymatic mechanism of the iodination of aromatic rings appears to be poorly understood.

SUMMARY

The dependence of the ratio of the thyroid radioiodide concentration to the serum radioiodide concentration (T/S ratio) on the serum iodide concentration was studied in male C_3H mice. On increasing the serum iodide concentration, starting at low levels, the T/S ratio remained constant until the binding of radioiodide began to be blocked. The T/S ratio then increased with the serum iodide concentration until the amount of iodide bound was small compared with the thyroid iodide content. As the serum iodide concentration was further increased, the T/S ratio progressively decreased. When binding was blocked by propylthiouracil and iodide the T/S ratio was approximately twice that when binding was blocked by iodide alone.

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THE INFLUENCE OF CORTICOTROPIN ON KETONEMIA AND GLYCEMIA IN NORMAL AND ADRENALECTOMIZED RATS

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PREVIOUS studies from this laboratory demonstrated that adrenalectomy and hydrocortisone or cortisone treatment of adrenalectomized and normal rats had opposing influences with respect to ketosis during a four day fast (Scott and Engel, 1953, Engel and Engel, 1954a). Thus, adrenal insufficiency was associated with enhanced ketonemia, provided the circulation was sustained with DCA, while cortisone treatment of the adrenalectomized rat restored ketonemia to normal. In the normal rat treated with cortisone or hydrocortisone fasting ketosis was completely abolished. In contrast, ACTH in a dose which increased adrenal weight and the blood sugar had no effect on fasting ketosis, although it did depress the exaggerated ketonemia associated with exposure to a cold environment. This apparent discrepancy between the action of ACTH and cortisone was puzzling but in keeping with the contradictory results recorded in the literature. Thus, Kinsell *et al.* (1951) have described a suppression of fasting ketosis by corticotropin in man, Bennett *et al.* (1948 a,b) and Segaloff and Many (1952) reported a stimulating effect on fasting ketosis in the rat, while Bondy and Wilhelmi (1950) could elicit no effect of this hormone on ketonemia. The latter observers also were unable to demonstrate any influence of corticotropin treatment on acetoacetate production by liver slices while Hunter (1953) found a significant increase in ketone production by liver slices of rats after 10 days of corticotropin treatment.

These obvious contradictions in the literature as well as in our own experience led to the present reinvestigation of the role of corticotropin in ketone metabolism. Soon after the initiation of this study it became apparent that there were a number of unexpected non-endocrine variables influencing ketone metabolism in the rat which had to be isolated before the re-

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sults of corticotropin injection could be interpreted. These variables include peripheral circulatory failure even when mild (Engel and Hewson, 1953), respiratory hypoxia, sympathetic discharge, hyper- or hypothermia in anesthetized rats and even anesthesia itself (Engel and Hewson, 1954b). Anesthetized rats were found to be very much more sensitive to ketogenic hormones than were unanesthetized animals. For this reason both anesthetized and unanesthetized animals were used in these experiments. In the former special attention had to be given to the maintenance of a normal body temperature since both hypo- and hyperthermia modified the degree of ketosis observed. Both intact and adrenalectomized rats were studied, the latter because of the suspicion that the ketosis observed in response to certain corticotropin preparations might indicate either an extra-adrenal effect or, more logically, a contamination of corticotropin with a pituitary ketogenic factor.

METHOD

The source of the rats, their size, diet and handling and the analytical methods were the same as in previous reports (Scott and Engel, 1953, Engel and Engel, 1954a). Unanesthetized and anesthetized rats fasted overnight were used. The former were lightly restrained by wrapping them in a towel during bleeding from the cut tail. They did not struggle during this procedure. For anesthesia, nembutal was injected intraperitoneally at a dose of 4 mg./100 gm. for induction with supplements sufficient to maintain light anesthesia for the $3\frac{1}{2}$ hour period of observation. Adrenalectomy was performed by the lumbar approach under nembutal anesthesia 24 hours prior to the ketone studies, the animals receiving substitution therapy as indicated in the protocols. All experiments with anesthetized rats were carried out at least once in a ventilated constant temperature chick incubator set at 33° C., the environmental temperature found optimal for maintenance of the body temperature at a normal level in anesthetized rats (Engel and Engel, 1954b). Where the results checked closely ($p. > 1$) with those previously obtained from rats kept warm simply by exposure to an electric lamp they were pooled to give larger samples for intergroup analyses. Where the data did not correspond or when the earlier groups exhibited a wide scatter, presumably because of poorly controlled environmental factors, only the groups kept in the incubator were used for the intergroup analyses. Records were kept of the initial and final rectal temperatures of the rats in the incubator in order to be sure that body temperature was being kept constant.

The following hormone preparations were used: Cortisone acetate (Cortone Acetate, Merck) made up to a concentration of 5 mg./ml. of saline vehicle and administered at a dose of 0.5 or 1.0 mg./day for maintenance of adrenalectomized rats; desoxycorticosterone acetate in oil, 5 mg./ml.; cortisone aldehyde, 10 mg./ml. of 5% dextrose; corticotropin (Wilson, lot #84364), the regular commercial preparation at a concentration of 40 U.S.P. units/ml. of phenol water and assaying 1 unit/mg.; oxycel corticotropin (Wilson lots 82002 and 86890). These were special preparations of corticotropin which had been further purified on oxycel and assayed approximately 33 U.S.P. units/mg. They were supplied as water clear solutions containing 40 units/ml. of phenol water. A supply of the phenol water vehicle was obtained from the manufacturer for use in a control group of animals to establish that the phenol itself had no influence on ketosis. Oxycel purified corticotropin supplied by Dr. E. B. Astwood and assaying approximately

TABLE 1. ACUTE EFFECTS OF CORTICOTROPIN AND CORTISONE ALDEHYDE ON BLOOD KETONES IN FASTED UNANESTHETIZED RATS

Treatment	No. of rats	Dose,		Blood ketones, mg. % acetone†			P*
		units	mg.	Initial	3.5 hours	Difference	
Control	19	—	—	3.89±0.36	3.89±0.30	0.00±0.19	
ACTH (Wilson's Com. 84364)	4	0.4	0.4	4.04±0.47	4.34±0.47	0.30±0.27	N.S.
ACTH (Wilson's Com. 84364)	6	10	10.0	4.72±0.81	6.76±0.93	+2.05±0.57	<.02
ACTH (Wilson's Com. 84364)	14	20	20.0	4.18±0.32	7.36±0.32	+3.18±0.49	<.01
ACTH (Wilson's Com. 84364)	6	40	40.0	3.63±0.35	6.70±0.76	+3.07±0.51	<.01
ACTH (Wilson's—82002) Oxycel	6	1	0.03	2.91±0.22	3.25±0.28	+0.34±0.32	N.S.
ACTH (Wilson's—82002) Oxycel	3	10	0.3	3.17±0.27	2.88±0.42	−0.29±0.26	N.S.
ACTH (Wilson's—82002) Oxycel	6	20	0.6	3.77±0.43	4.17±0.53	+0.40±0.58	N.S.
ACTH (Wilson's—86890) Oxycel	15	10	0.3	3.83±0.31	3.93±0.26	+0.10±0.23	N.S.
ACTH (Wilson's—86890) Oxycel	6	20	0.6	2.18±0.26	2.67±0.23	+0.49±0.25	N.S.
ACTH—Astwood—Oxycel	4	8.8	0.11	3.34±0.47	4.06±0.70	+0.71±0.31	N.S.
ACTH—Astwood—Oxycel	4	160	2.0	3.99±0.49	5.02±0.46	+1.03±0.40	N.S.
ACTH—Astwood—Oxycel	8	240	3.0	4.76±0.30	9.59±1.36	+4.90±1.12	<.01
Cortisone aldehyde	6	—	1.0	3.52±0.32	4.19±0.36	+0.67±0.12	<.01

* Compared to corresponding control.
† Mean ± standard error.

80 U.S.P. units/mg. For the smallest doses used 1.5 mg. of the Astwood corticotropin was suspended in 5.0 ml. of phenol water, brought into solution with 0.1 N HCl and adjusted to pH 8.0 with 0.1 N NaOH. For the larger doses the hormone was dissolved at concentrations of 2 and 3 mg. respectively in 0.25 ml. of phenol water and adjusted to pH 5.5. with 0.1 N HCl. At higher pH levels the hormone began to come out of solution.

RESULTS

Unanesthetized rats. Table 1 records the acute effects of the several different corticotropin preparations and of cortisone aldehyde on fasting ketosis in unanesthetized rats. The corticotropin was injected intraperitoneally in doses ranging between 0.03 and 40 mg. (0.4 to 240 U.S.P. units depending on the preparation), and the cortisone aldehyde at a dose of 1 mg. Blood samples were taken prior to and at 3.5 hours after injection.

The control group showed no change in blood ketones in 3.5 hours. Corticotropin stimulated ketosis only when injected in comparatively large doses. The minimal effective dose tested was 10 mg. (10 units) of Wilson's commercial corticotropin and 3 mg. (240 units) of the Astwood oxycel corticotropin. No dose of either of the Wilson oxycel corticotropins tested was active, but the maximal dose which could be used conveniently without injecting undue volumes of solution was 0.6 mg. (20 units). It is apparent from these results that there is no correlation between the doses of corticotropin in U.S.P. units and in milligrams and the response with respect to ketonemia. After cortisone aldehyde there was a small, but statistically significant increase in ketones. It is questionable, however, whether an increase of this magnitude has biological significance.

Anesthetized rats. As can be seen in Table 2, the response of the rat anesthetized with nembutal is quite different from that of the unanesthetized animal and is characterized by a marked increase in sensitivity to the ketogenic hormone. Furthermore, nembutal anesthesia per se is associated with some stimulation of ketosis as is apparent in the 3.5 hour rise of 2.66 ±0.28

TABLE 2. ACUTE EFFECTS OF CORTICOTROPIN AND CORTISONE ALDEHYDE ON BLOOD KETONES IN FASTED ANESTHETIZED RATS

Treatment	No. of rats	Dose of ACTH,		Blood ketones, mgm. % acetone†			P*
		units	mg.	Initial	3.5 hours	Difference	
Control	19	—	—	4.78±0.44	7.44±0.61	+2.66±0.28	—
ACTH (Wilson Com. 84364)	9	0.1	0.1	4.36±0.28	8.24±0.52	+3.88±0.44	<.05
ACTH (Wilson Com. 84364)	12	0.4	0.4	3.92±0.39	7.79±0.66	+3.87±0.46	<.05
ACTH (Wilson Com. 84364)	7	10.0	10.0	4.44±0.68	10.78±1.66	+5.97±0.67	<.01
ACTH (Wilson—82002) Oxycel	8	10.0	0.3	4.11±0.91	9.02±1.45	+4.91±0.69	<.01
ACTH (Wilson—82002) Oxycel	6	20.0	0.6	3.38±0.25	8.86±0.42	+5.35±0.26	<.01
ACTH (Wilson—86890) Oxycel	6	0.1	0.003	3.35±0.28	6.32±0.93	+2.98±0.70	N.S.
ACTH (Wilson—86890) Oxycel	7	1.0	0.03	3.77±0.44	11.29±1.01	+7.52±1.12	<.01
ACTH (Wilson—86890) Oxycel	32	10.0	0.3	4.36±0.24	10.81±0.80	+6.85±0.64	<.01
ACTH—Astwood—Oxycel	3	0.2	0.003	4.20±0.33	6.31±0.95	+2.11±0.62	N.S.
ACTH—Astwood—Oxycel	3	2.4	0.03	4.36±1.44	10.08±2.01	+5.73±0.63	<.01
ACTH—Astwood—Oxycel	4	4.8	0.06	4.37±0.67	12.76±1.37	+8.39±1.05	<.01
Cortisone Aldehyde	12	—	1.0	3.69±0.36	5.87±0.54	+2.18±0.30	N.S.

* Compared to control.
† Mean ± S.E.

mg. % in the control animals. This reaction does not appear to be unique for nembutal anesthesia as it has been observed in rats anesthetized with other agents (Engel and Engel, 1954b). Although the mechanism of this response is still obscure, it has served the useful purpose of simplifying the comparison of the ketogenic activity of different pituitary hormone preparations.

All samples of corticotropin tested were active in causing a significant ketosis in 3.5 hours with the minimum effective dose tested in each case being 0.1 mg. (0.1 unit) for commercial corticotropin, 0.03 mg. (1.0 unit) for Wilson's oxycel corticotropin and 0.03 mg. (2.4 unit) for the Astwood oxycel corticotropin. The Wilson and Astwood oxycel corticotropins thus appeared to be equivalent in ketogenic activity but not in corticotrophic activity, although this conclusion must be tempered by the variability which may exist in the corticotropin assay. In any case, the dose of oxycel corticotropin which was ketogenic was exceeding small and according to the reports in the literature (Bennett *et al.*, 1948) as well as our own experience considerably less than the effective ketogenic dose of growth hormone. Cortisone aldehyde in these experiments had no effect on ketonemia.

Further experiments demonstrated that when it occurs the ketogenic response to corticotropin is exceedingly rapid, being already well established 30 minutes after the injection of the hormone into anesthetized rats (Fig. 1).

Blood sugar changes. Figure 2 records data on the blood sugar changes in corticotropin treated rats. In the control anesthetized rat there is an immediate rise in the blood sugar at 30 minutes followed by a gradual fall towards the starting level by 3.5 hours. The rats receiving 0.3 mg. oxycel corticotropin (Wilson) exhibited much less blood sugar rise at 30 minutes while at 60 minutes the blood sugar levels were distinctly depressed and had not yet returned to normal by 3.5 hours. The unanesthetized rats

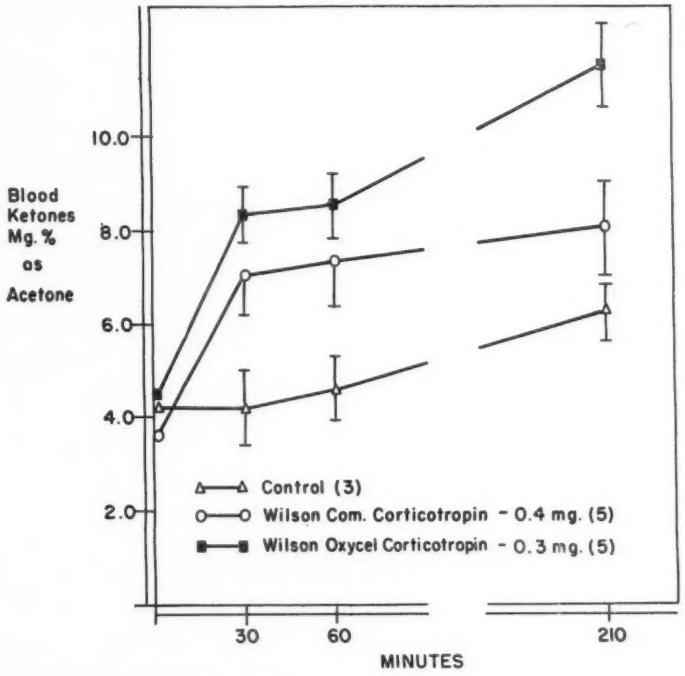

FIG. 1. Immediate effect of an intraperitoneal injection of Wilson's commercial and oxycel purified corticotropin on blood ketone levels of 24 hour fasted rats anesthetized with nembutal.

showed a sustained rise in blood sugar on bleeding and this was not modified by 0.3 mg. of Wilson oxycel corticotropin which, it will be recalled (Table 1) did not effect ketosis. Injection of 20 units (20.0 mg.) of Wilson's commercial corticotropin, which induced ketonemia in the unanesthetized rat, was followed by an immediate brisk rise in blood sugar at 30 minutes, which then rapidly declined towards but never below the initial value. It should be noted that the injection of large doses of this particular hormone was associated with definite evidences of distress in the normal rats and with collapse and death in adrenalectomized rats. The data of Figures 1 and 2 clearly show that neither a fall in blood sugar nor hypoglycemia per se is a necessary prerequisite to ketosis. Indeed, the ketone change preceded the blood sugar fall. Nevertheless in many studies there was an exceedingly good correlation between the fall in blood sugar and the rise in blood ketones. This is shown in a striking fashion in Figure 3 which is a scattergram of the blood sugar and ketone changes at 3.5 hours in unanesthetized rats receiving varying doses of Astwood oxycel corticotropin. In general, the

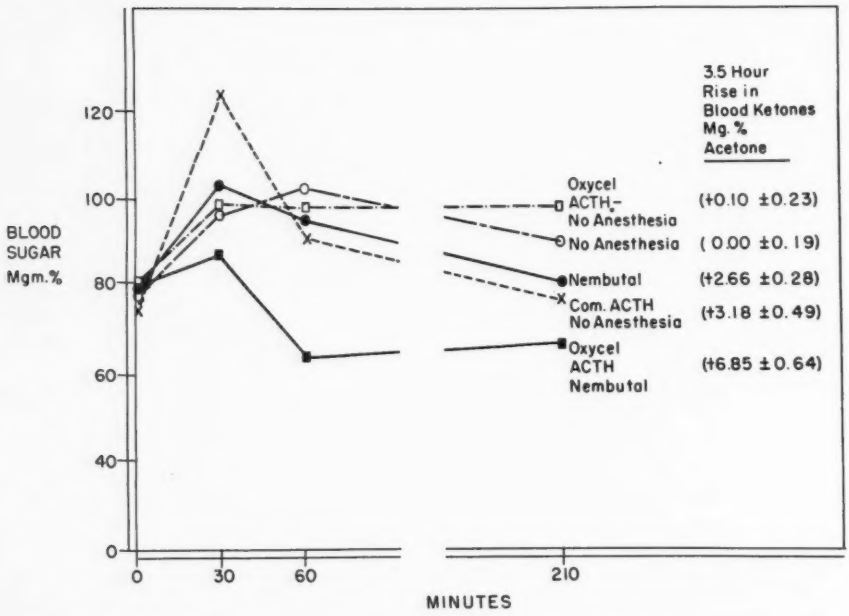

FIG. 2. Immediate effects on the blood sugar of an intraperitoneal injection of corticotropin in 24 hour fasted anesthetized and unanesthetized rats. The oxycel corticotropin was a Wilson preparation injected at a dose of 0.3 mg. (10 U.S.P. units). The dose of the commercial corticotropin in unanesthetized rats was 20 mg. (20 U.S.P. units).

correlation was good regardless of dosage for the occasional large dose of corticotropin which failed to cause ketosis also did not lower the blood sugar.

Anesthetized, adrenalectomized rats. A final series of experiments was performed on cortisone and DCA maintained adrenalectomized rats in order to determine whether or not the ketogenic response to oxycel corticotropin (Wilson) was mediated by its adrenocorticotrophic action and if not whether its occurrence was dependent on the presence of adrenal steroids. Compared to normal rats under nembutal anesthesia the adrenalectomized rats receiving either 0.5 or 1.0 mg. of cortisone acetate or 0.5 mg. of DCA daily for 3 days all showed less ketosis during the $3\frac{1}{2}$ hour period (Table 3). This contrasts with the normal and greater than normal ketosis respectively in fasting cortisone and DCA maintained unanesthetized adrenalectomized rats (Engel and Engel, 1954a). The difference no doubt relates to the greater tendency of the adrenalectomized animal to develop respiratory obstruction with hypoxia and peripheral circulatory failure when un-

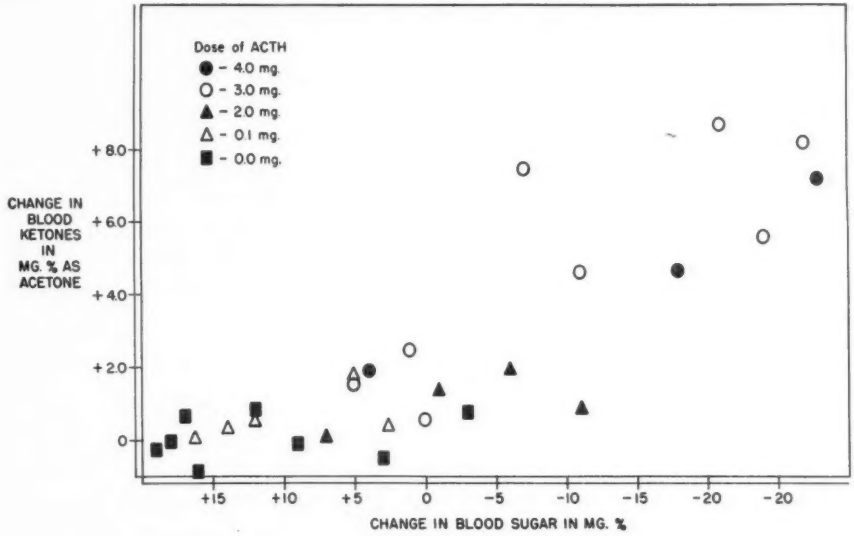

FIG. 3. Relationship between blood ketone and sugar changes in 3½ hours in unanesthetized rats receiving varying doses of Astwood oxycel corticotropin intraperitoneally.

der barbiturate anesthesia with consequent suppression of ketosis (Engel and Hewson, 1953). Both the cortisone and the DCA maintained rats exhibited significant ketosis ($p < .01$) after treatment with oxycel corticotropin, but a considerably larger dose of hormone was required than in the normal animal. 0.3 mg. (10 units) was ineffectual while 0.6 mg. (20 units)

TABLE 3. ACUTE EFFECTS OF CORTICOTROPIN† ON BLOOD KETONES AND SUGARS IN FASTED ANESTHETIZED ADRENALECTOMIZED RATS

Treatment	No. of rats	Dose of ACTH, units	Dose of ACTH, mg.	Blood ketones, Mg. % acetone° Initial	3.5 hours	Difference	P*
A. Adrx—0.5 mgm. Cortisone	17	—	—	3.84±0.24°	5.54±0.49	+1.70±0.36	—
B. Adrx—0.5 mgm. Cortisone	13	10	0.3	4.02±0.41	6.66±0.80	+2.64±0.56	N.S.
C. Adrx—0.5 mgm. Cortisone	18	20	0.6	3.96±0.28	8.91±0.71	+4.96±0.51	<.01
D. Adrx—1.0 mgm. Cortisone	8	—	—	3.79±0.34	4.86±0.55	+1.07±0.29	—
E. Adrx—1.0 mgm. Cortisone	13	20	0.6	3.82±0.39	8.37±0.80	+4.52±0.55	<.01
F. Adrx—0.5 mg. DCA	17	—	—	4.78±0.22	6.61±0.41	+1.83±0.35	—
G. Adrx—0.5 mg. DCA	10	20	0.6	4.07±0.28	7.17±0.44	+3.10±0.36	<.01
				Blood Sugar, Mgm. %			
A	14	—	—	78±1.6	70±2.2	− 8±2.6	—
B	3	10	0.3	78±5.6	54±4.5	−24±1.1	<.01
C	19	20	0.6	71±2.2	49±1.7	−22±1.5	<.01
D	4	—	—	63±4.3	58±2.1	− 5±3.1	—
E	7	20	0.6	74±4.7	53±3.5	−21±4.8	<.01
F	6	—	—	69±3.1	56±3.9	−13±2.6	—
G	10	20	0.6	63±2.6	49±2.6	−14±3.7	N.S.

° Mean ± Standard Error.
* Compared to corresponding control.
† Oxycel Corticotropin (Wilson—86890).

induced a significant increase in adrenalectomized rats. This compares to the minimal effective dose of 0.03 mg. in intact rats. Nevertheless, it is still apparent from these experiments that the ketogenic response is not dependent on the presence of the adrenal cortex or of adrenal hormone.

All cortisone-maintained adrenalectomized rats receiving 0.6 mg. oxycel corticotropin showed a significant fall in blood sugar compared to their corresponding controls. Blood sugars were measured in only 3 rats receiving 0.3 mg. oxycel corticotropin, a dose which did not induce ketosis. The fall in these 3 animals was the same as in previous groups. In the DCA treated rats there was no difference in blood sugar fall between the controls and the corticotropin treated animals.

DISCUSSION

The data outlined in this report as well as others to be published emphasize some of the difficulties and pitfalls in the study of ketosis in the rat. The results, nevertheless help considerably in reconciling some of the contradictions in the literature as well as in our own experience. It is apparent that certain preparations of corticotropin will induce ketosis in either unanesthetized or anesthetized rats while others will do so only in anesthetized rats. In the study of Bennett *et al.* (1948a,b) in which ketosis was described after ACTH the rats were anesthetized with evipal. Bondy and Wilhelmi (1950), who found no ketonemic response to corticotropin, used unanesthetized rats. Segaloff and Many (1951) recorded ketonuria after administering 20–40 units of ACTH to unanesthetized phloridzinized rats but noted that the same preparation of ACTH proved lethal to adrenalectomized rats. The response in this case might correspond to our own results with Wilson's commercial corticotropin. The present study has thus reproduced all these experiences. The report of Kinsell *et al.* (1951) showing a suppression of fasting ketosis in normal humans who were receiving ACTH by prolonged continuous infusion is understandable in terms of the intense stimulation of adrenal steroid secretion which is to be expected under these circumstances, since he and ourselves have shown that cortisone and hydrocortisone inhibit fasting ketosis. The experimental conditions in Kinsell's study contrast to those of the present investigation in which the corticotropin was administered as a single large intraperitoneal injection, a technique less conducive to the stimulation of a brisk and sustained adrenal cortical secretory response.

These studies also demonstrate clearly that the ketogenic response to corticotropin, when it occurs, is not mediated through the adrenal cortex since it persists, albeit to a lesser degree, in the adrenalectomized rat. Nor does it seem to be dependent on the presence of 11 oxy steroids, judging

from the response of the DCA maintained adrenalectomized rat. It cannot be determined from these experiments whether the fact that twenty times as much corticotropin is required to elicit ketosis in adrenalectomized rats is indicative of a lesser sensitivity of these rats because of adrenal insufficiency per se or because of some degree of peripheral circulatory failure secondary to nembutal anesthesia.

Further evidence against the ketogenic response to corticotropin being mediated by the adrenal cortex is found in the fact that the ketonemia is already well established one-half hour after injection, an unusually rapid metabolic response for the pituitary-adrenal axis. And finally, cortisone aldehyde, a water soluble, rapidly absorbed cortisone preparation did not produce a comparable response. This finding, of course, is not surprising in light of our previous experience with cortisone and hydrocortisone in fasting ketosis (Scott and Engel, 1953, Engel and Engel, 1954a).

Recently a number of reports have been published from the laboratory of E. B. Astwood (Astwood, Raben, Payne and Grady, 1951; Astwood, Raben, Rosenberg and Westermeyer, 1953; Rosenberg, 1953; and Westermeyer and Raben, 1954) concerning the metabolic effects of an oxycel-purified corticotropin in the mouse. Although the oxycel corticotropin originally used in our experiments was acquired from a different source (Wilson Laboratories) subsequent results with the preparation supplied by Dr. Astwood indicate that we are dealing with similar preparations. Astwood's group has found their material to be highly potent in adipokinetic activity, hypoglycemic activity and in producing acetonuria in the fasted mouse and in lowering the R.Q. in the fed mouse. All these effects persisted in the cortisone maintained adrenalectomized mouse while the blood-sugar lowering effect also took place in the saline maintained adrenalectomized mouse. In our study the ketonemic response was still demonstrable in the DCA maintained adrenalectomized rat. Hypoglycemia also occurred, but this was not easily interpreted in light of the comparable fall in the DCA control under nembutal anesthesia. It is noteworthy that the time of response and the size of the dose of oxycel corticotropin necessary to produce ketosis in rats in our experiments were comparable to those of Astwood in mice. In both cases the oxycel corticotropin appeared to be active at a considerably lower dose level than that of the less purified corticotropin preparations or purified growth hormone. Under conditions comparable to those in the present study we have found the minimum effective dose of several different growth hormone preparations to range between 0.2–5 mg. in anesthetized rats. Whether the same principle is active in oxycel corticotropin and in growth hormone remains to be established. The complete lack of correlation between the dose of corticotropin in milligrams

versus that in U.S.P. units strongly suggests that the ketogenic effect is not a function of the same molecular configuration responsible for ascorbic acid depletion of the adrenal cortex.

The finding of a number of metabolic activities obviously not mediated through the adrenal cortex in a hormone preparation which was previously assumed to act only through its target gland was unexpected until the divergent effects of cortisone and corticotropin on ketosis were noted. At that time the suspicion was born that the effects of corticotropin on ketosis might represent the response to a non-corticotropic activity in the hormone preparation. This was borne out by the results in adrenalectomized rats, the lack of correspondence between the response and the dose in units of ACTH activity and subsequently by the published reports of Astwood and coworkers. Further significance is attached to the results by the comparable finding of Ingle, Nezamis, Morley and Li (1953) that certain highly purified preparations of corticotropin at low dosage improved the work performance of adrenalectomized-hypophysectomized rats if they were maintained with adrenal cortical extract.

In our experiments, the possibility is not ruled out that the effects on ketonemia and glycemia are mediated by the endogenous secretion of a pituitary metabolic factor in the injected animal. The observations of Ingle above as well as those of Astwood *et al.* (1953) in hypophysectomized rats suggest that this is not the case.

The present studies throw no new light on the mechanism by which the pituitary factor induces ketosis. The fall in blood sugar in association with the ketonemia is a provocative finding, as already commented upon by Lotspeich and Petersen (1954) in their studies on growth hormone. Although there often was a good correlation between these two phenomena the ketonemia preceded the fall in blood sugar and sometimes even occurred in the absence of a blood sugar fall. This suggests that the blood sugar changes are not a reflection of metabolic changes playing a causal role in ketosis.

Further information is needed and is currently being sought in this laboratory concerning the mechanism by which nembutal anesthesia so markedly increases the sensitivity of the rat to the ketogenic effect of pituitary extracts. The magnitude of this sensitization is most apparent in the experiments with Astwood oxycel corticotropin where the minimal effective dose in unanesthetized rats was one hundred times that in the anesthetized animal. Factors which are being considered include: (1) A direct effect of the anesthetic on the liver metabolism of acetate favoring ketogenesis. (2) Supression of epinephrine discharge secondary to handling and bleeding. Observations in this laboratory have shown that injection of

epinephrine lowers blood ketone levels in the rat. (3) A decrease in the rate of peripheral utilization of ketone bodies by the resting muscle of the anesthetized rat.

SUMMARY

1. Blood ketone and sugar levels were measured for 3.5 hours after intraperitoneal injection of several different preparations of corticotropin and of cortisone aldehyde into 24 hour fasted normal and adrenalectomized rats, with and without nembutal anesthesia.

2. In unanesthetized rats, only comparatively large doses of corticotropin induced ketonemia (10–40 U.S.P. units in the case of Wilson's commercial corticotropin and 3 mg. or 240 units in the case of Astwood oxycel corticotropin). There was no correlation between ketogenic and ACTH activity *per se* since 10–20 U.S.P. units of another oxycel purified corticotropin, weighing 0.3–0.6 mg. had no ketogenic activity. Cortisone aldehyde did not influence blood ketones in unanesthetized rats.

3. In anesthetized rats all samples of corticotropin tested were ketogenic, some in minute doses, i.e. 30 micrograms (1 U.S.P. unit of Wilson oxycel purified corticotropin and 2.4 U.S.P. units of Astwood oxycel purified corticotropin). Ketonemia was well established within 30 minutes of injection. Cortisone aldehyde had no appreciable effect on ketonemia in 3.5 hours.

4. The ketogenic response to oxycel purified corticotropin was associated with a concurrent fall in blood sugar, beginning in about 60 minutes while that after commercial corticotropin (Wilson) was not. In the former cases there was a good correlation between the rise in ketones and the fall in blood sugar.

5. The ketogenic response to oxycel purified corticotropin persisted in DCA or cortisone maintained adrenalectomized rats, although twenty times more hormone than in normal rats was necessary to elicit it in the absence of the adrenal.

6. Ketonemia in the adrenalectomized rats was associated, in general, with a fall in blood sugar.

7. The ketogenic response to corticotropin is interpreted as an extra-adrenal response. It appears to be independent of ACTH activity per se, as expressed in units by the adrenal ascorbic acid depletion method, and hence presumably represents a separate principle.

8. The relationship between these results and the previous contradictory reports in the literature on the effect of corticotropin on ketone metabolism is discussed.

Acknowledgments

We are much indebted to Dr. E. B. Astwood, Boston, Massachusetts and to Dr. David Klein, The Wilson Laboratories, Chicago, Illinois, for generous supplies of oxycel

and commercial corticotropin and to Dr. Elmer Alpert, Merck and Company for cortisone acetate and cortisone aldehyde.

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CYTOLOGIC ALTERATIONS IN THE ANTERIOR PITUITARY GLAND FOLLOWING THYROIDECTOMY: AN ELECTRON MICROSCOPE STUDY[1]

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INTRODUCTION

IN THE course of a detailed study of the cytology of the anterior pituitary gland as revealed by electron microscopy, a series of rats were subjected to thyroidectomy. It soon became evident that alterations of a distinctive character occurred in certain of the basophils which served to distinguish them from the basophils stimulated by gonadectomy. The characteristic fine structural alterations which occur following gonadectomy have been described in detail (Farquhar and Rinehart, 1954). Examination of the early response to thyroidectomy has enabled us to recognize the unstimulated thyrotrophic cell in the normal animal. It is the purpose of this report to indicate the fine structural organization of this cell and its response to thyroidectomy as well as to contrast it with the gonadotroph of the normal animal and the castrate. The studies of Purves and Griesbach (1951a, b) and Halmi (1950 and 1952) have served importantly as guides in our recognition of the thyrotroph. These authors have shown that two basophils of the anterior pituitary of the rat can be distinguished on the basis of differences in form, position and staining properties. Thyrotrophic cells were found to be angular and concentrated chiefly in the central areas of the gland; they were usually not located on the portal vessels or sinusoids. Gonadotrophic cells, on the other hand, were rounded, chiefly present in the peripheral segments of the gland and typically aggregated along blood vessels. Both cells were stained with the periodic acid-Schiff technique, while only the thyrotrophic cell was stained with aldehyde-fuchsin.

With the electron microscope we have been able to distinguish these cells on morphologic features alone. Thus, our observations support the concept of Purves and Griesbach and Halmi that gonadotrophs (delta cells) and thyrotrophs (beta cells) are separate cell types.

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[2] Grateful acknowledgment is made to Mrs. Karin Taylor for technical assistance.

MATERIALS AND METHODS

Observations were made on the pituitaries of normal male and female rats of the Sprague-Dawley strain and on males which had been thyroidectomized at 60 days of age and sacrificed at intervals of 5, 10, 20 and 35 days after operation.

As the result of the application of improved techniques, our current electron micrographs are superior to those previously illustrated (Rinehart and Farquhar, 1953; Farquhar and Rinehart, 1954). The chief factors in improved techniques are: 1) the application of shorter fixation and dehydration times as suggested by Palade (personal communication); 2) the use of the Servall microtome, adapted after the design of Porter and Blum (1953), for thin sectioning. Further details of technical procedures are recorded elsewhere (Rinehart and Farquhar, 1954).

OBSERVATIONS

Thyrotrophs and Gonadotrophs of the Normal Rat Pituitary

As observed by Purves and Griesbach, and Halmi, we find the thyrotrophs in the normal rat pituitary to be angular and not usually located on a sinusoid. The cytoplasm contains variable numbers of fine, ill defined secretory granules (maximal diameter ± 140 mμ) (Fig. 2). The gonadotrophs are rounded, typically located on a sinusoid or larger portal vessel and contain somewhat larger, denser rounded granules (maximal diameter ± 200 mμ) (Fig. 1). Both cell types have rounded nuclei with one or several nucleoli and show variable numbers of ovoid vesicles in the cytoplasm in addition to the usual cytoplasmic constituents, i.e., mitochondria, small fluid droplets or vesicles, tiny particles (Palade, 1953) and occasional canalicular structures of the endoplasmic reticulum (ergastoplasm).

Thyroidectomy Basophils vs. Castration Basophils

Following thyroidectomy or gonadectomy these two types of basophils show additional differences in fine structure which serve to distinguish

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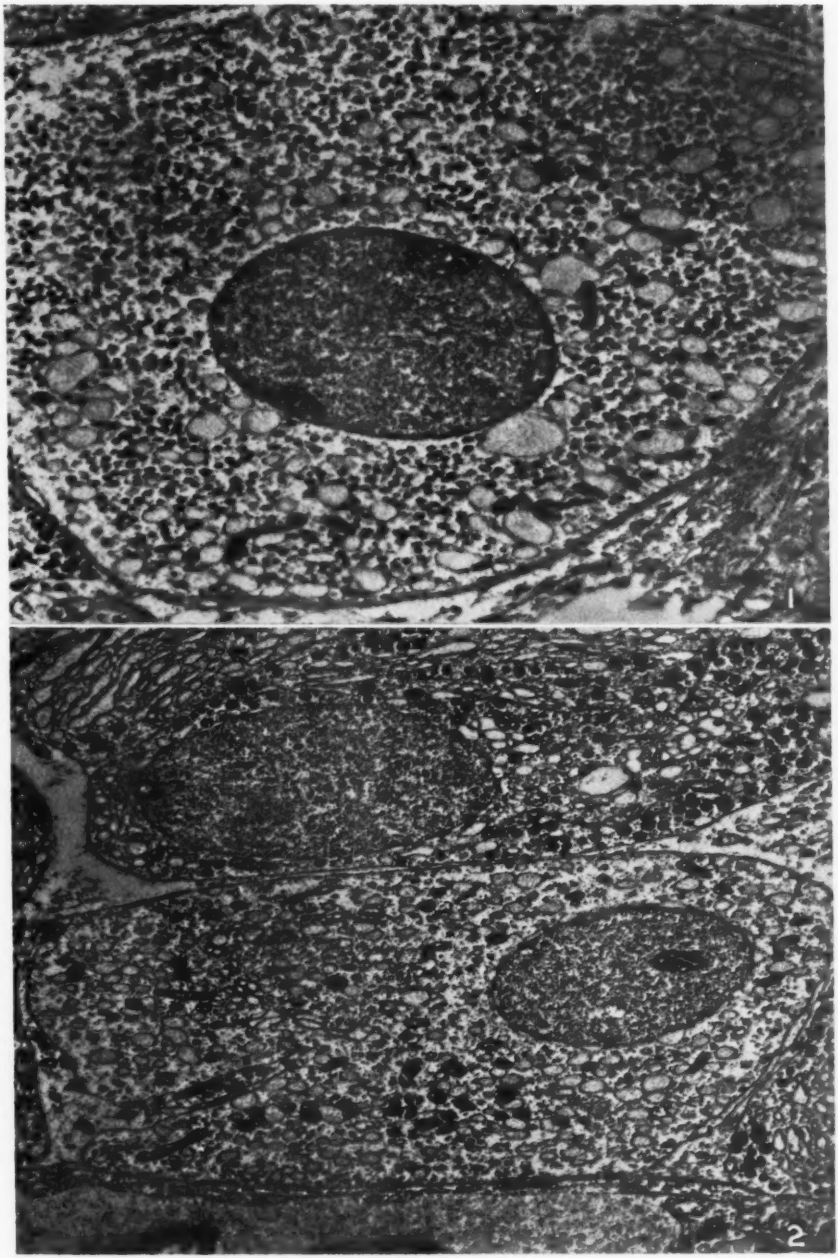
EXPLANATION OF FIGS. 1–2

FIG. 1. Gonadotroph from the pituitary of a normal 70 day old male rat. This cell has rounded contours and contains an ovoid nucleus with portions of two nucleoli. The gonadotroph cytoplasm is seen to contain many dense, rounded secretory granules (maximal diameter, ± 200 μ) and a few mitochondria which have a relatively dense background matrix. Numerous vesicles of varied sizes are also present in the cytoplasm. These vesicles exhibit a light though definite density. $\times 10{,}800$.

FIG. 2. Thyrotroph from the pituitary of a normal 60 day old male rat. This cell may be contrasted with the gonadotroph above. It also contains an ovoid nucleus with a nucleolus and cytoplasmic granules and vesicles. However, it is more elongated and, on the left, shows a cytoplasmic indentation. In addition, there are fewer secretory granules, and the granules are smaller (maximal diameter, ± 140 μ) than those of the gonadotroph. $\times 8100$.



FIGS. 1-2

them. The distinguishing features include differences in shape, grouping, vesicle size, granule content, form and density of the mitochondria and components of the Golgi apparatus, as indicated below:

As early as 5 days following thyroidectomy, increased numbers of vesiculated basophils are seen in the anterior pituitary gland. These "thyroidectomy cells" usually maintain an angular shape, are often grouped together and are usually not found adjacent to sinusoids (Figs. 4–8).

Perhaps the most striking features distinguishing thyroidectomy cells are related to the size and appearance of the cytoplasmic vesicles. In ovariectomized rats we are able to estimate the postoperative period by the size of the vesicles in the stimulated pituitary basophils. The vesicles of the castration cell do not vary much in size within the cell (Fig. 3), from cell to cell or in cells of different glands. The average vesicle size is small in early castrates and later gradually increases by a coalescence of smaller vesicles into larger, more irregular units. On the other hand, the vesicles of thyroidectomy cells are in general of a larger size for a given period following operation. The size is also much more variable both within the same cell (Figs. 4, 6, 8) and from cell to cell (Figs. 5 and 6; 7 and 8). While one thyroidectomy basophil may contain small vesicles, a neighboring cell may contain quite large vesicles or a mixture of large and small vesicles.

Another feature which makes the picture so different following thyroidectomy is the difference in the degree of the thyroidectomy changes from gland to gland, presumably associated with the amount of thyroid tissue which was left at operation. An estimate of the remaining, functioning thy-

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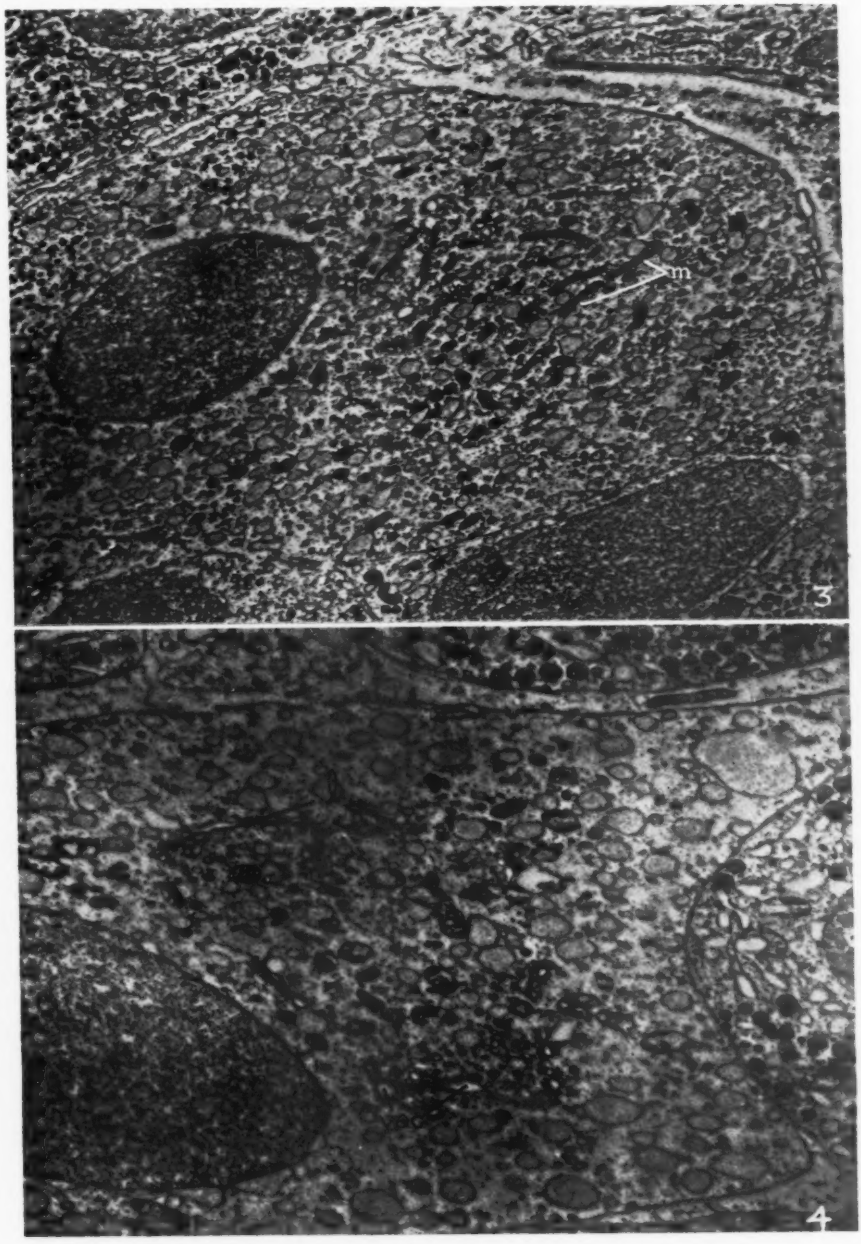
EXPLANATION OF FIGS. 3–4

FIG. 3. Castration basophil from a 20 day castrate. This stimulated gonadotroph is larger than those of the normal animal. It retains its rounded contours. The cytoplasm contains some secretory granules and many vesicles which are relatively uniform in size. The Golgi apparatus (ga) occupies the portion of the cytoplasm to the right of the nucleus. It is represented chiefly by a beadlike chain of clear vacuoles with associated denser lamellar structures. The mitochondria are concentrated in the Golgi zone. A few long filamentous mitochondria (m) are seen along with the more numerous short rods. The background matrix of the mitochondria is relatively dense. ×7200.

FIG. 4. Thyroidectomy basophil from a pituitary 5 days after thyroidectomy. This cell shows the irregular contour which characterizes thyrotrophic basophils. In contrast to the castration basophil above, the cytoplasm contains very few secretory granules, and the vesicles, even at this early stage, are seen to vary considerably in size. A large Golgi apparatus is seen to the right of the nucleus. It consists of a differentiated zone of cytoplasm outlined by electron-dense lamellar structures (l) and closely associated vacuoles (vac) with a very low electron-scattering power. The mitochondria have a lighter background matrix than those of the castration basophil above. In addition, the mitochondria are shorter, short rods and spheres predominating. ×8100.



FIGS. 3–4

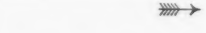
roid tissue can be made on the basis of the granule content of acidophils within a given gland (Lebedewa, 1936; Purves and Griesbach, 1946).

The variability in appearance of the thyroidectomy cells from gland to gland and within the same gland makes it difficult to describe a typical picture for a given period following the operation. However, in general it may be said that the number and prominence of the vesiculated cells and the average size of the vesicles increase with time after thyroidectomy. In addition, the process is most advanced in those animals in which thyroidectomy was most complete, as estimated by the greatest reduction in the granule content of acidophils. As previously pointed out by Halmi (1950), it is interesting that numerous vesiculated thyroidectomy cells are present even in those animals where greater numbers of acidophil granules remain. However, we have noted that the process of vesiculation is not as advanced as it is in the glands showing lesser numbers of remaining acidophil granules.

Another feature characteristic of thyroidectomy cells is the relative paucity of secretory granules. In castration cells, as late as 75 days postoperatively some secretory granules of the characteristic size remain in the cytoplasm between the large vesicles. However, following thyroidectomy, very few typical granules are seen, even in "early" thyroidectomy cells containing smaller vesicles (Figs. 4 and 5). In the cells showing the greatest numbers of large vesicles, *no* secretory granules can be seen (Figs. 7 and 8).

The finding of uniformly dense, rounded bodies *within* the vesicles of thyroidectomy cells is of particular interest. These structures are seen as early as 10 days following thyroidectomy and increase in size and number as the period after operation increases. At 10 days after thyroidectomy only an occasional cell contains such structures (Fig. 9). By 30 days, the majority of the thyroidectomy cells show a few or many of these dense bodies (Figs. 7 and 8). Comparable structures have not been seen in castration cells. Their nature and possible significance will be discussed in a later section.

The Golgi bodies of the thyroidectomy cells are well defined and are somewhat enlarged. The Golgi apparatus is recognizable as a differentiated



EXPLANATION OF FIG. 5

FIG. 5. Section from the pituitary of an animal 10 days after thyroidectomy. The thyroidectomy cell (T) is seen to contain few (if any) secretory granules in the vesiculated cytoplasm. The mitochondria (m) are mostly spherical, but a few short rods are present. The small gonadotroph (G) seen above is readily distinguished from the thyroidectomy cell. This gonadotroph is smaller than those seen in the normal rat. The cytoplasm contains many secretory granules. The mitochondria (m) have a denser matrix than those of the thyroidectomy cell and are, for the most part, longer rods or filaments. The Golgi apparatus (ga) of the thyroidectomy cell occupies a much larger zone of the cytoplasm than the Golgi apparatus of the gonadotroph. ×7200.

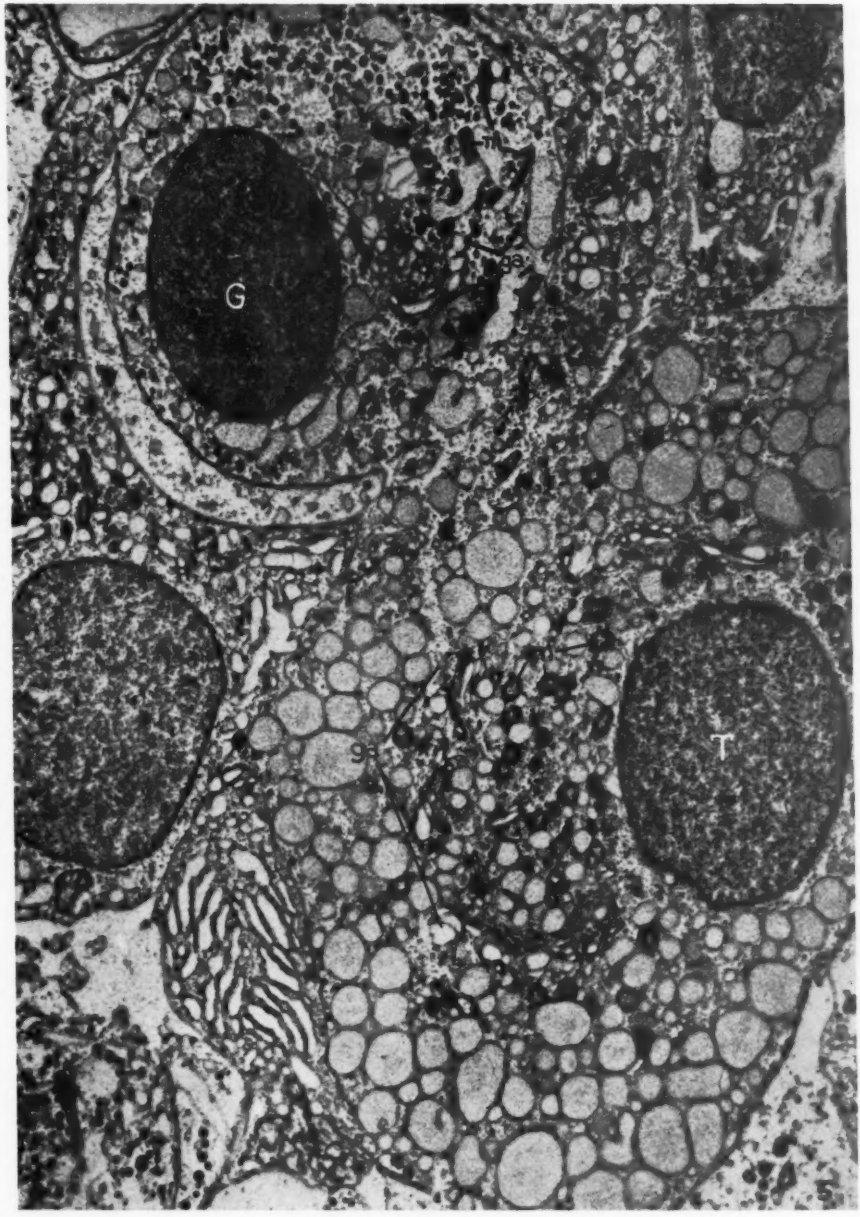


FIG. 5

area of cytoplasm outlined by lamellar structures with little or no internal density and electron-dense walls (Figs. 4 and 10). Vacuoles are frequently seen in intimate association with the fine lamellar structures. Aggregated around the lamellar structures and vacuoles are many tiny droplets or vesicles. The area of the cytoplasm outlined by the lamellar structures shows many small particles, fine fluid droplets or clear vesicles, and mitochondria, but does not contain as many nor as large "colloid"-filled vesicles. The Golgi apparatus of the thyroidectomy cell differs from that of the castration cell by having a less conspicuous vacuolar element. The castration cell shows a somewhat similar differentiated cytoplasmic zone in which are concentrated many mitochondria, granules, particles and vesicles, but the zone is outlined by coarser vacuoles arranged in a circular or s-shaped, beadlike chain.

The mitochondria of thyroidectomy cells are conspicuous and are usually oval or spherical in shape. They are found in the Golgi area and in the strands of peripheral cytoplasm that separate the vesicles (Figs. 4–6). The long filamentous mitochondria, commonly found in castration basophils, are rarely seen.

While the nuclei of the thyroidectomy and castration cells are similar, they do show certain differences. Both are ovoid and contain one or several nucleoli. The nucleoli of the thyroidectomy cells are somewhat larger than those of the castration basophils when cut in the plane of their greatest diameter. One feature sometimes encountered in thyroidectomy cells, which has not been seen in castration cells, is an indentation of the nucleus which appears to arise as the result of pressure from an adjacent, enlarging cytoplasmic vesicle.

Acidophils

All of the thyroidectomized animals examined show a striking degranulation of the acidophils. Heavily granulated or moderately granulated acidophils are infrequently seen. Most commonly, only a few (perhaps one or two up to one dozen) granules may be visible in the sectioned cell (Figs. 9

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EXPLANATION OF FIG. 6

FIG. 6. Higher magnification showing detail of a 10 day thyroidectomy basophil. The cytoplasm is essentially filled with cytoplasmic vesicles which vary a great deal in size and shape. Note that secretory granules are *not* present. An area of high electron-scattering power associated with the lamellar element of the Golgi apparatus (ga) is seen above the nucleus. Most of the mitochondria (m) are spherical although a few short rods are present. They are seen in the Golgi area and in the strands of cytoplasm remaining between vesicles. ×10,000.

FIG. 6

and 11). The cytoplasm shows numerous expanded and coarsened canalicular structures of the endoplasmic reticulum and mitochondria which are mostly spheres or short rods. The mitochondria characteristically have a very light background matrix. Some of the mitochondria are of normal size and shape and contain a well developed system of internal ridges or "cristae mitochondriales" (Palade, 1952, 1953a). However, others appear swollen, are of irregular shapes and contain less intricate or flattened internal "cristae" (Fig. 11).

Gonadotrophs

The gonadotrophs of the thyroidectomized animal appear to be smaller than the gonadotrophs of the normal animal. However, they retain their previous form and position and are readily distinguished from adjacent thyroidectomy cells (Fig. 5).

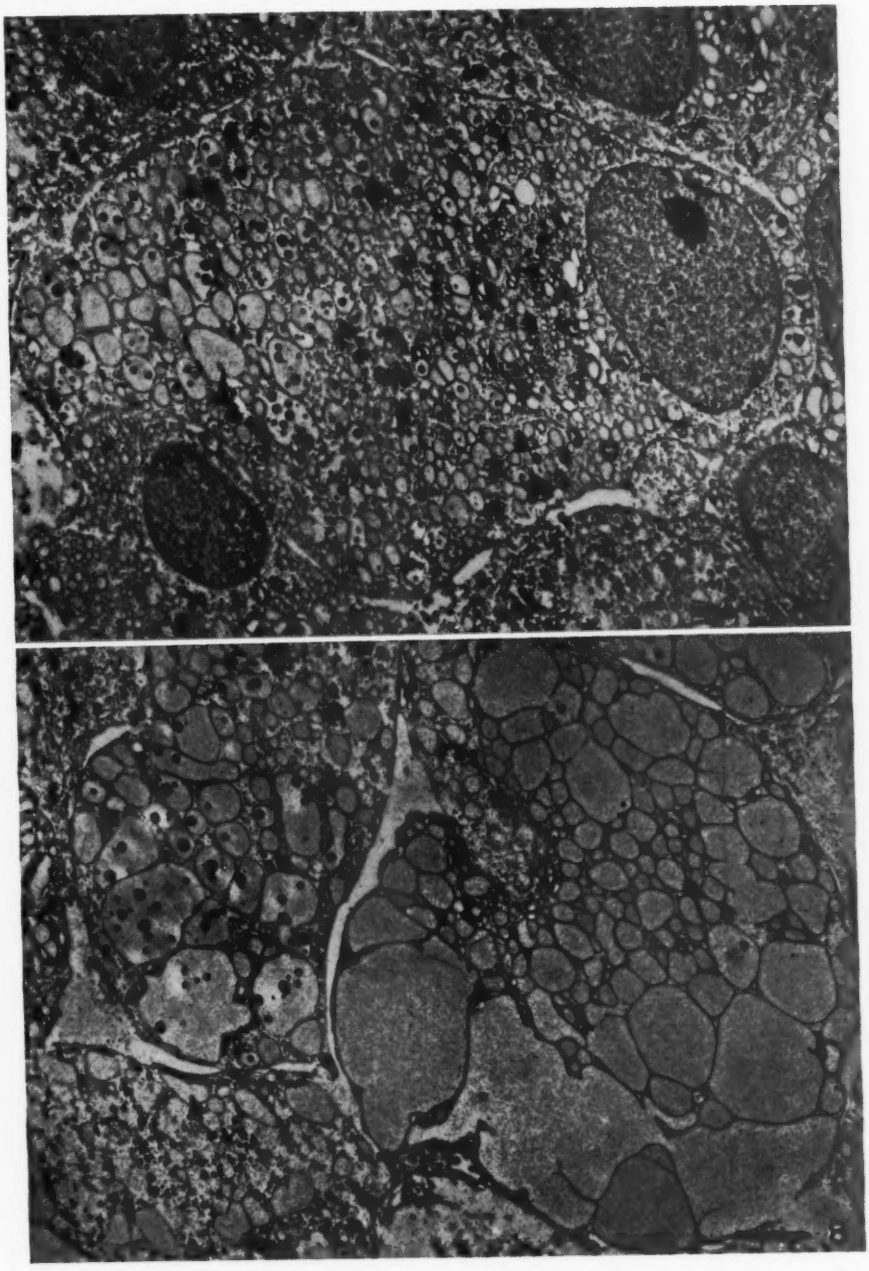
Secretory Products in the "Perisinusoidal Space"

In a separate communication dealing with the fine vascular organization of the anterior pituitary gland (Rinehart and Farquhar, 1954) we noted that the sinusoids are surrounded by an actual or potential "space" which lies between the endothelium and parenchymal cells and is outlined by two basement membranes. The perisinusoidal space is of relative light density. In addition to the perisinusoidal "reticulo-endothelial" cells found here, *this space frequently contains secretory vesicles, granules and portions of the cytoplasm of both acidophils and basophils.*

The perisinusoidal spaces of thyroidectomized animals often contain acidophilic granules or segments of cytoplasm of acidophils. Of particular interest is the occasional finding of thyroidectomy cells which appear to be in direct communication with the "space" and releasing segments of cytoplasm into it.

EXPLANATION OF FIGS. 7–8

FIGS. 7 and 8. These two figures illustrate cells from the pituitary of an animal sacrificed 35 days after thyroidectomy. A nucleolus is prominent within the nucleus of the thyroidectomy basophil above. The size of the cytoplasmic vesicles varies a great deal from cell to cell and within the same cell. Conspicuous dense, rounded or ovoid bodies are present within many of the cytoplasmic vesicles. They are seen either singly or aggregated into groups and are much denser than the mitochondria. The thyroidectomy basophil of Figure 7 contains a great number of these dense bodies, but the cell on the left of Figure 8 contains fewer numbers, and the cell on the right of Figure 8 shows only a half dozen or so. These structures are undoubtedly the same as those described by Purves and Griesbach and Halmi as being associated with long term thyroid deficiency. Fig. 7, ×5400; Fig. 8, ×5000.



FIGS. 7–8

DISCUSSION

The observations set forth have shown that thyrotrophs and thyroidectomy cells possess characteristic morphologic features which distinguish them from gonadotrophs and castration cells. Some of these features deserve comment in view of previous studies and in regard to functional implications of fine cytologic structure.

Zeckwer *et al.* (1935) first reported that thyroidectomy and castration basophils differ morphologically. Later Zeckwer (1938) in a convincing study indicated that thyroidectomy and castration basophils differ functionally. Guyer and Claus (1937) and Reese, Koneff and Wainmann (1943) outlined a number of criteria which could be used to distinguish thyroidectomy and castration basophils. Among the distinguishing features which these authors have described and which our observations confirm are the tendency for grouping, greater irregularity in size of the cytoplasmic vesicles and greater size attained by thyroidectomy cells.

By utilizing the observations set forth, certain speculations can be made relating staining with the periodic acid-Schiff (PAS) technique and aldehyde-fuchsin (AF) to specific structural elements. Catchpole (1949) examined PAS-stained sections prepared by freeze-drying technique from pituitaries of rats which had been thyroidectomized for 24 days. He found that the glands contained "amounts of glycoprotein granules in basophils comparable with the castrated series." Purves and Griesbach (1951a) found that during the first three to four weeks following thyroidectomy "the reaction with PAS (in the thyrotrophs) is only slight. A pink color due to fine granulation can be seen." They also noted that "hyaline vacuoles appearing at the second or third week stain darker than the cytoplasm and, on the whole, darker than the castration cell vacuoles." The difference in the results of these two groups of workers can probably be attributed to

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EXPLANATION OF FIG. 9

FIG. 9. Field from the pituitary of an animal 10 days after thyroidectomy. The acidophils (A) are seen to be practically depleted of secretory granules. The acidophil cytoplasm contains prominent, relatively "coarsened" canalicular structures (can) of the endoplasmic reticulum. The mitochondria (m) are large and have a light background matrix.

Portions of thyroidectomy basophils are visible in the field to the right above, and in the lower right corner. Both cells contain characteristic, rounded, dense structures within cytoplasmic vesicles. Portions of two granulated gonadotrophs (G) are present.

The row of canalicular structures (can) in the thyroidectomy cell in the lower right corner is of particular interest. These structures may be seen to communicate with the cytoplasmic vesicles and, in several places, dilatations of the canalicular chain appear to be forming vesicles. ×5100.

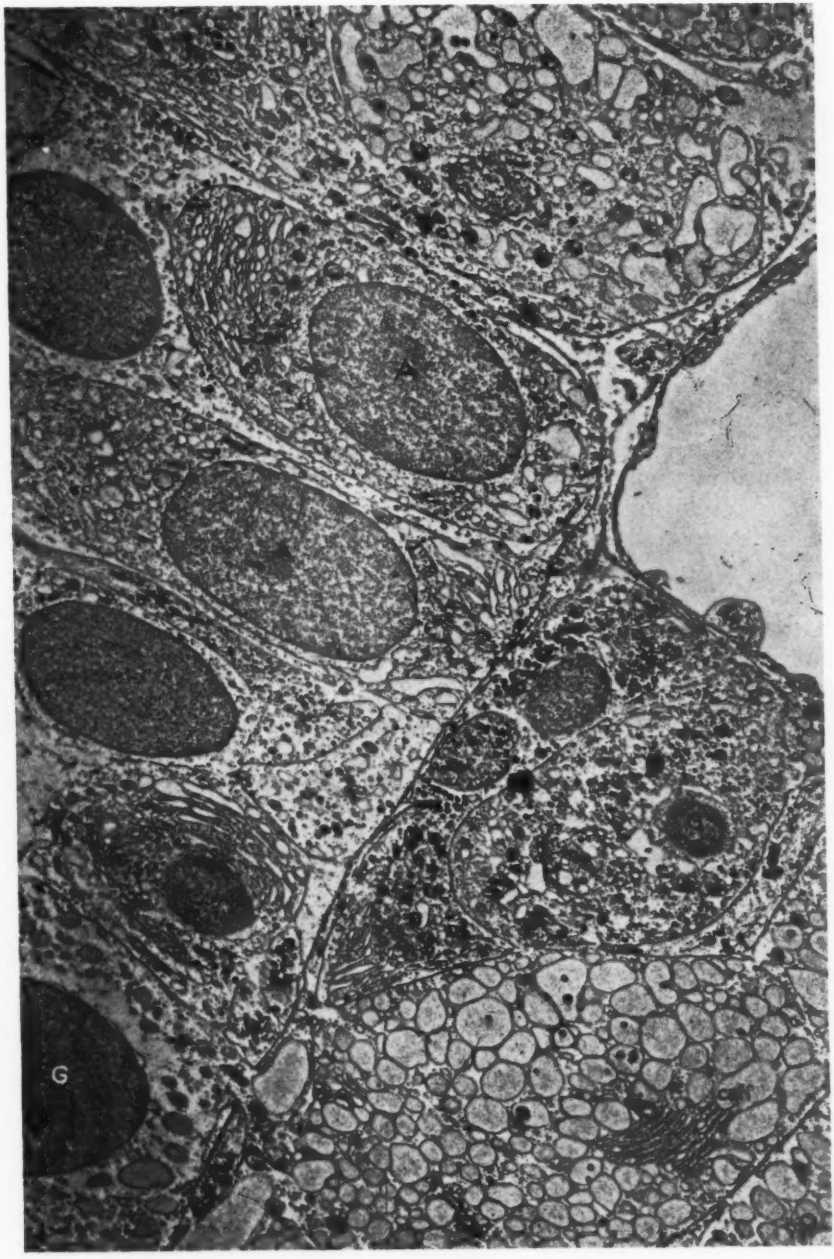

FIG. 9

differences in techniques. However, they both found that PAS staining was maintained following thyroidectomy. On the other hand, Purves and Griesbach (1951b) and Halmi (1952) found that AF staining of these cells was diminished following thyroidectomy.

We believe that the PAS staining reaction is related to the vesicles and that the AF staining is related to the granules of the thyrotrophs. The reasons for this belief are: 1) thyrotrophs of the normal animal are both PAS and AF-positive, and electron micrographs show that they contain both secretory granules and vesicles; 2) thyroidectomy cells are PAS-positive, but staining with AF is diminished and, in electron micrographs, it is evident that vesicles are larger and more numerous than in the normal thyrotroph, but the granule content is diminished or virtually absent.

It seems likely that the dense, osmiophilic spherical or ovoid bodies which we see within cytoplasmic vesicles of thyroidectomy basophils are the same structures which Purves and Griesbach (1951a) and Halmi (1952) described in thyroidectomy cells. Purves and Griesbach found that, in PAS-stained glands 4 to 6 weeks after thyroidectomy, a large number of thyroidectomy cells contain dark red particles in their cytoplasm which are of varied sizes and shapes. Halmi noted that these same particles stain with AF only after prolonged oxidation with hydrogen peroxide. All of these authors relate these large bodies to long term thyroid deficiency. However, we have seen them as early as 10 days following thyroidectomy. Because we find these large bodies ordinarily within vesicles, we believe that they may represent condensations of altered material stored within the large vesicles.

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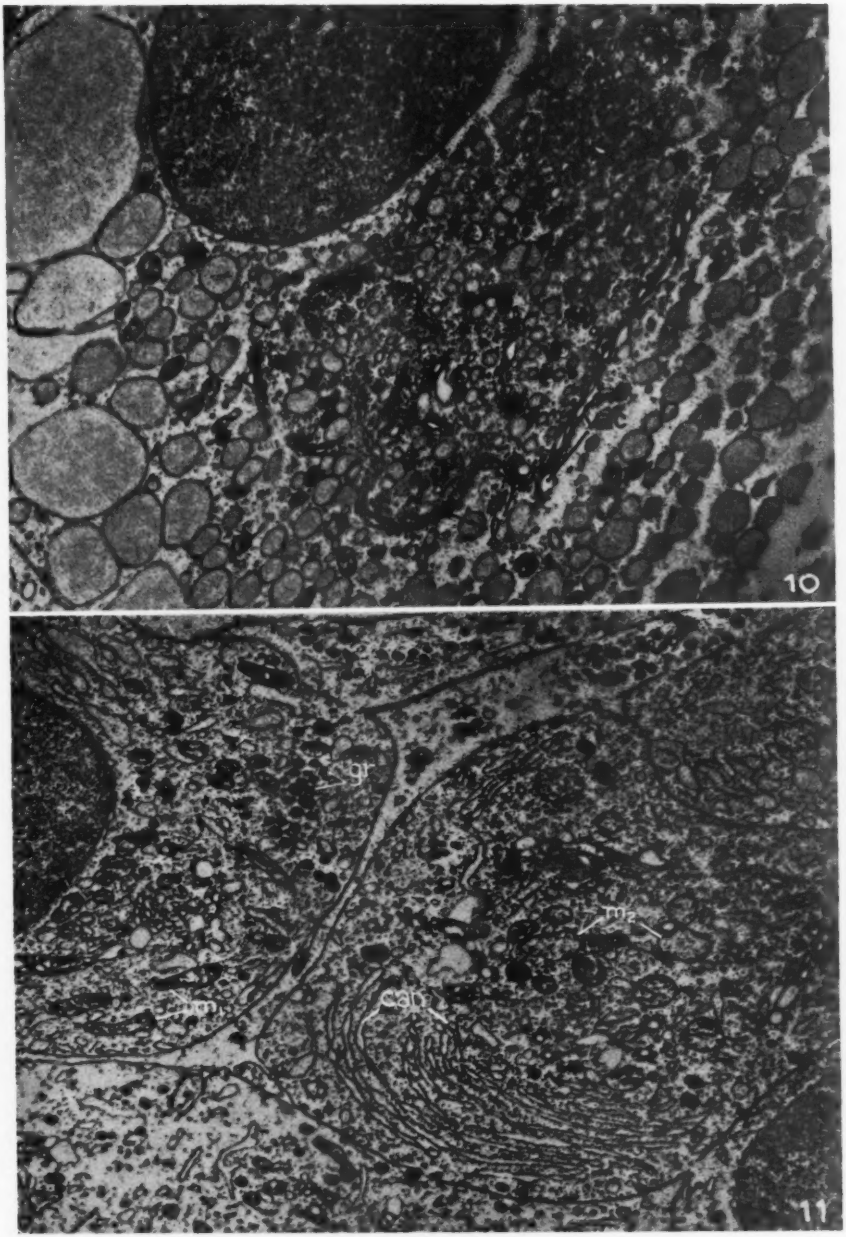
EXPLANATION OF FIGS. 10–11

FIG. 10. Portion of a 10 day thyroidectomy basophil. The large Golgi apparatus below the nucleus is seen to consist of a differentiated zone of cytoplasm outlined by electron-dense lamellar structures (l) and closely associated vacuoles (vac) with a low electron-scattering power. Many small fluid droplets or vesicles, particles, and mitochondria are concentrated in the area circumscribed by the lamellar structures. The colloid-containing vesicles in the Golgi area are much smaller than those in the surrounding cytoplasm. ×8100.

FIG. 11. Portions of the cytoplasm of several acidophils from the pituitary of an animal thyroidectomized for 10 days. Very few secretory granules (gr) are seen within the cytoplasm. The cytoplasm contains small vesicles and numerous canalicular structures (can) of the endoplasmic reticulum with closely associated fine particles. The alterations of the mitochondria are of particular interest. Some (m_1) are filamentous, of normal size and contain well developed internal ridges or cristae mitochondriales of Palade, while others (m_2) appear swollen, are of irregular shapes and contain only a few flattened "cristae." ×9000.



FIGS. 10–11

Canalicular Structures of the Endoplasmic Reticulum (Ergastoplasm) and Vesicles

We have noted (Rinehart and Farquhar, 1953) that fine canaliculi of the type seen in the pituitary acidophils (and the pancreatic acinar cells, parenchymal cells of the liver, etc.) are infrequently found in the basophils of the anterior pituitary gland. However, variable numbers of ovoid vesicles are characteristically present in the cytoplasm of basophils. We also noted (Farquhar and Rinehart, 1954) that, following castration, the basophils in the earlier phases of vesiculation show what appear to be transitions between slightly expanded canaliculi and vesicles. We suggested that the vesicles of the basophils may represent expansions of the double-layered canaliculi between points of constriction and may serve to store hormonal products.

Our observations on thyroidectomy basophils substantiate our previous findings. We have occasionally seen the walls of the canalicular structures of the endoplasmic reticulum to be continuous with the walls of vesicles. In certain instances vesicles unquestionably are seen to represent expansions of the double-layered canaliculi between points of constriction (Fig. 9).

Golgi Apparatus

In earlier communications (Rinehart and Farquhar, 1953; Farquhar and Rinehart, 1954) we briefly reviewed pertinent literature concerning the Golgi apparatus, particularly as applicable to the anterior pituitary gland and in regard to information contributed by electron microscopic studies. At this time a re-examination of the Golgi apparatus seems appropriate, for improved techniques have allowed a much more satisfactory examination of this specialized cytoplasmic zone.

Dalton and Felix (1954) have quite recently described three morphologically distinct components of the Golgi substance of the cells of the rat epididymis seen with the electron microscope. These are: 1) large vacuoles, 2) small "granules" ($\pm$400 Å) with a relatively low electron-scattering power and 3) lamellae with electron-dense walls ($\pm$70 Å), separated by an area ($\pm$70 Å) of low electron-scattering power. Our observations indicate that these three structural units are present in the Golgi bodies of the pituitary gonadotrophic and thyrotrophic basophils. We would prefer to use the term "Golgi vesicles" rather than granules, because the bodies have a central zone of lesser density, and reserve the term "granule" for particles of uniform density and high electron-scattering power.

We have noted that the vacuoles are more conspicuous in the Golgi apparatus of the castration basophil, and the lamellar constituent is more prominent in the thyroidectomy basophil.

Mitochondria

We have previously suggested (Farquhar and Rinehart, 1954) that the size, shape, density and internal organization of mitochondria may vary considerably with the functional activity of the cell.

Reese, Koneff and Wainman (1943) noted that the mitochondria of thyroidectomy cells are large and are usually seen as spheres or short rods. In contrast to the mitochondria of castration basophils, true filaments are rarely (if ever) seen. In addition, these authors noted that the mitochondria of thyroidectomy basophils do not show the same tendency for aggregation in the Golgi area as is characteristic of castration cells. We have found that the mitochondria of thyroidectomy cells have a lighter background matrix and a simpler system of internal ridges than the castration cell.

The mitochondria of the acidophils of thyroidectomized animals are of particular interest. It has been pointed out that they are mostly spheres or short rods and sometimes become quite large. In addition, it was noted that the background matrix is very light, and the internal ridges are simpler than in the normal. Since the acidophils are generally believed to be depressed following thyroidectomy, it is probable that these mitochondrial changes reflect lowered functional activity. It may be that these mitochondrial changes seen in the acidophils following thyroidectomy represent a characteristic alteration of this structure reflecting the depressed functional activity of cells.

Effects of Thyroidectomy on the Acidophils

It is well known that degranulation of the acidophils follows thyroidectomy. It is also known that an animal stops growing soon after thyroidectomy (Evans *et al.*, 1939). Since thyroxine has little or no effect on growth in the hypophysectomized animal (Smith, 1927; Evans *et al.*, 1939; Ray *et al.*, 1950, and others), it seems likely that the influence of thyroxine on growth is mediated through the secretion of hypophyseal growth hormone (Scow *et al.*, 1949; Koneff *et al.*, 1949 and Becks *et al.*, 1950). The recent work of Eartly and Leblond (1954) further indicates that, in the absence of thyroxine, the hypophysis secretes little or no growth hormone.

Since the acidophil is generally considered to be the site of production of growth hormone, the evidence summarized above indicates that acidophil activity is suppressed. It might, therefore, be assumed that the structural appearance of the cell, i.e., swollen mitochondria, expanded canalicular structures, lack of granules, etc. reflects this lowered functional activity.

COMMENT

The observations reported here and the survey of a very large number of electron micrographs from the pituitary glands of animals subjected to

a variety of experimental procedures bring increasing conviction that cells responsible for the formation of different trophic hormones possess distinctive features of fine structure. Thus, among the basophils the gonadotrophic and the thyrotrophic cells appear to have characteristic fine structural differences. In addition, we have presented evidence supporting the existence of two morphologically distinct gonadotrophs (Farquhar and Rinehart, 1954). Our own additional studies (Farquhar and Rinehart, unpublished data) and those of Siperstein *et al.* (1954) add support to the concept of a "FSH-gonadotroph" and an "LH or ICSH gonadotroph." Further, we have made some observations which indicate that the cell which elaborates the adrenocorticotrophic hormone also possesses a characteristic structure. Our observations on the cell which we believe to be responsible for formation of the adrenocorticotrophic hormone will be described in a subsequent report.

Some progress is being made in relating variations in fine structure of mitochondria, the Golgi apparatus, endoplasmic reticulum, etc. to states of functional activity of cells of the anterior pituitary gland.

SUMMARY

Thin sections of the anterior pituitary gland of young thyroidectomized and control rats were examined with the electron microscope.

Examination of early thyroidectomy changes established definitive morphologic criteria which allowed recognition of the unstimulated thyrotrophic cell in the normal animal. The thyrotroph is seen to be angular and to contain fine, ill-defined secretory granules. It is usually not located directly on a sinusoid. These features of the thyrotroph are contrasted with those of the gonadotroph which is more rounded, contains larger, denser granules and is typically located on a sinusoid. Both cell types exhibit rounded nuclei with one or two nucleoli and show variable numbers of ovoid vesicles in the cytoplasm in addition to the usual cytoplasmic constituents, i.e., mitochondria, fine particles, small fluid droplets or vesicles and a few canalicular structures of the endoplasmic reticulum (ergastoplasm).

Following thyroidectomy, changes are seen to occur in the fine structure of the thyrotrophs which further distinguish them from normal gonadotrophs and gonadotrophs stimulated by castration. Thyroidectomy basophils are ordinarily grouped together and contain larger vesicles at earlier periods following operation. The size of the cytoplasmic vesicles is seen to vary a great deal from cell to cell and from gland to gland. In general, however, the average vesicle size increases with time following thyroidectomy. Thyroidectomy cells contain few or no secretory granules and those present are small and ill defined. As early as 10 days following thyroidectomy, uniformly dense ovoid or rounded bodies are found within cytoplasmic vesi-

cles. These bodies increase in size and number as the period following operation increases.

The morphologic features characteristic of thyroidectomy basophils are contrasted with those of the castration basophils. The latter show no tendency for grouping, contain more uniform vesicles for a given period following operation and always contain well defined secretory granules. The dense rounded bodies characteristically seen in the vesicles of thyroidectomy cells are not found in castration cells.

The observations are related, insofar as is possible, to earlier morphologic studies and more recent histochemical investigations on thyroidectomy and castration basophils.

Variations noted in the mitochondria, Golgi apparatus and canalicular structures of the endoplasmic reticulum (ergastoplasm) are described and discussed.

Our findings fully confirm previous studies indicating that acidophils become degranulated following thyroidectomy. The significance of this is briefly discussed, and the fine structure of the functionally depressed acidophil is described.

The observations presented indicate that thyrotrophic and gonadotrophic basophils can be distinguished on morphologic features alone. Our observations support the concept presented by Purves and Griesbach and Halmi that gonadotrophs and thyrotrophs are separate cell types.

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1955 LAURENTIAN HORMONE CONFERENCE

The 1955 Laurentian Hormone Conference will be held at Estes Park, Colorado, during the period September 11–16, 1955. This Western meeting has been instituted particularly to enable attendance at the meeting by qualified investigators who have found it difficult to attend the conferences at an Eastern site.

In view of a need for detailed advance arrangement, interested investigators should make application to the Committee on Arrangements of The Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 6, 1955. Attendance at the Conference is limited by the accommodations available. A Conference rate of $12.00 per person per day is extended to all invited participants. Final announcement of the Conference program will be made early in 1955.

ANNOUNCEMENT

The Division of Medical Sciences, National Academy of Sciences–National Research Council, is accepting applications for grants-in-aid of research in the following fields:

The Committee for Research in Problems of Sex is concerned with encouraging research, primarily on the mechanisms controlling sexual behavior in animals and man. Proposals involving endocrinological, neurological, psychological, anthropological, phylogenetic, and genetic studies directed toward this objective are therefore invited. Requests will also be considered that deal with the physiology of reproduction or with related biological and biochemical fields. Applications for the fiscal year 1955–1956 should be postmarked on or before February 15, 1955.

ANNOUNCEMENT OF THE VAN METER PRIZE AWARD

The American Goiter Association again offers the Van Meter Prize Award of Three Hundred Dollars and two honorable mentions for the best essays submitted concerning original work on problems related to the thyroid gland. The award will be made at the annual meeting of the Association which will be held in Oklahoma City, Oklahoma, April 28, 29 and 30, 1955, providing essays of sufficient merit are presented in competition.

The competing essays may cover either clinical or research investigations; should not exceed three thousand words in length; must be presented in English, and a typewritten double space copy in duplicate sent to the Secretary, John C. McClintock, M.D., 149½ Washington Avenue, Albany, New York, not later than January 15, 1955.

THE FRANCIS AMORY SEPTENNIAL PRIZE
OF THE
AMERICAN ACADEMY OF ARTS AND SCIENCES

In compliance with the terms of a gift under the will of Francis Amory, the American Academy of Arts and Sciences offers a substantial prize for outstanding work addressed to the alleviation or cure of diseases affecting the human reproductive organs, in particular those of men. The work may be instrumental technology, operative procedure, clinical or laboratory research, or a technical treatise. No formal applications and no essays or treatises from candidates are desired; but nominations, supported by statements of qualifications in respect to work consummated between November 10, 1947, and November 10, 1954, will be given careful consideration. Address:

American Academy of Arts and Sciences
28 Newbury Street
Boston 16, Massachusetts

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By

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Associate Clinical Professor of Medicine
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